

8-2012

The role of acetyl-CoA carboxylase in the survival of *Trypanosoma brucei* during infection

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THE ROLE OF ACETYL-COA
CARBOXYLASE IN THE SURVIVAL OF
Trypanosoma brucei DURING
INFECTION

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Ciara Anderson McKnight
August 2012

Accepted by:
Dr. Kimberly S. Paul, Committee Chair
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ABSTRACT

This master's thesis focuses on how disruption of fatty acid metabolism affects both host adaptation and immune evasion in the deadly eukaryotic parasite, *Trypanosoma brucei*. In Chapter 1, I review the current literature on African trypanosomiasis, fatty acid synthesis, immune evasion, and lipid metabolism. In Chapter 2, I investigate how disruption of the fatty acid synthesis pathway affects the parasite's ability to evade the host immune defenses. When *T. brucei* acetyl-CoA carboxylase (TbACC) is knocked down by RNA interference (RNAi), fluid phase and receptor mediated endocytosis pathways are greatly affected, suggesting that fatty acid synthesis is necessary for both endocytic pathways. Further, the parasite's ability to clear surface immune antigens was reduced under ACC-RNAi conditions. In Chapter 3, I begin to elucidate the connection between fatty acid synthesis, fatty acid uptake, and lipid storage. ACC-RNAi caused a significant reduction in the number of lipid droplets. This effect can be reversed by the addition of external fatty acids, suggesting that lipid droplets are possibly a sorting site for both internally synthesized fatty acids as well as fatty acids taken up from the environment. Therefore, fatty acids are believed to be internalized to the ER, transferred to the lipid droplet, and then incorporated into the fatty acid synthesis pathway, where they can be utilized within the cell or returned to the lipid droplet. These studies serve as a preliminary linkage between fatty acid metabolism and host-parasite interaction, which is needed to fully understand *T. brucei* and bring forth a new cure.

DEDICATION

To my family and friends:

My extremely supportive parents, grandparents, and in-laws, who were always loving and understanding.

My loving husband Jared for his constant encouragement and support.

Rosie and Lola for their valuable discussion throughout my entire degree program.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Kim Paul for giving me the opportunity to work in her lab and the trust and guidance to work independently on each project we pursued.

Additionally, I would like to thank the members of my committee, Drs. Jim Morris and Lesly Temesvari. I appreciate your thoughtful discussion and time, while serving on my committee.

I would also like to thank Dr. Meredith Morris and Dr. Patrick Vigueira for their patience and guidance. They were paramount in teaching me the techniques used throughout this manuscript. I would also like to thank the members of the Morris Lab, Temesvari Lab, Turnbull Lab, and McNealy Lab for helpful discussion and continued support.

Last but not least, I would like to thank Sunayan Ray and the undergraduates of the Paul lab, Meredith, Ben, Audrey, Tiffani, Mohammed, and Christina for their understanding, hardwork, and excellent companionship.

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CHAPTER ONE

LITERATURE REVIEW

AFRICAN TRYPANOSOMIASIS

African trypanosomiasis is caused by the early branching eukaryotic parasite, *Trypanosoma brucei*. The parasite is transmitted to the mammalian host through the bite of the tse tse fly. There are 3 subspecies and a closely related species that infect either humans or livestock, such as cattle. The two subspecies that infect humans are *T. brucei gambiense* and *T. brucei rhodesiense*. *T. b. gambiense* is found on the western side of the Nile rift valley, accounts for 95% of infections, and causes a chronic infection leading to death in approximately 3 years. *T. b. rhodesiense* is found on the eastern side of the Nile rift valley, accounts for 5% of infections, and causes an acute infection, where death can occur in as little as 3 weeks (Brun *et al.*, 2010).

African trypanosomiasis also attributes to significant livestock loss; the resulting disease, nagana, causes wasting disease in cattle. It is estimated that ~\$4.5 billion dollars are lost annually due to nagana. The infectious subspecies of livestock include *T. brucei brucei* and the related species, *T. congolense*. Humans are not infected by *T. b. brucei* because all humans possess a high density lipoprotein, trypanolytic factor (TLF), which serves as part of our innate immunity. TLF lyses the parasite when it is taken up via endocytosis by the parasite within the human bloodstream (Wheeler, 2010). Therefore, *T. b. brucei* is used in laboratory settings as a study organism.

Approximately 6.2 million square miles of sub-Saharan Africa is at risk for the fatal disease in humans (FAO, Food and Agricultural Administration of the United Nations, 2007). From 1998-2004, there were an estimated 50,000-70,000 cases annually (WHO, 2012). Subsequently, since 2009 and the WHO's implementation of alternative treatment methods, such as insect repellent and nets, that number has declined to under 10,000 reported cases annually (WHO, 2012). However, the number of annually reported cases is likely a low estimate due to civil unrest and the general lack of infrastructure, which plagues much of sub-Saharan Africa. In some regions, the rate of human African trypanosomiasis (HAT) or sleeping sickness remains especially high, causing greater morbidity and mortality than HIV/AIDS or malaria (WHO, 2012).

HAT manifests itself in two distinct clinical stages. Early-stage infections are characterized by fever, swollen lymph nodes, and headache, if symptoms are even present. The parasites are limited to the bloodstream and lymph during this stage. Late-stage infections are far more severe and are characterized by worsening symptoms, including severe insomnia and disruptions in circadian rhythms, causing a lethargic coma-like state, lending to the origin of its more common name, "sleeping sickness." Parasites in late-stage infections have crossed the blood-brain barrier and are present in the central nervous system (brain and cerebral spinal fluid (CSF)) (Brun, *et al.*, 2010).

***T. brucei* Lifecycle**

T. brucei alternates between two hosts, the mammal and the tse tse fly. The parasite is transmitted to the mammal from an infected tse tse fly's bite. The mammalian bloodstream form parasites (BFs) live extracellularly in the bloodstream, where they proliferate by binary fission. BFs can cross the blood-brain barrier, resulting in coma and eventually death. The BFs remaining in the bloodstream differentiate into non-proliferative "short stumpy" forms. These "short stumpy" forms are primed to re-enter the tse tse fly.

When a tse tse fly bites an infected mammal, it picks up the "short stumpy" form parasites in the blood meal. These parasites will differentiate into the proliferative procyclic forms (PFs) and undergo a short proliferation stage in the midgut. Then, they exit the midgut, traversing the midgut wall, where they travel through the hemolymph to the salivary glands. Once in the salivary glands, there is a developmental change to the metacyclic form. This is the form that will be passed to the mammalian host, thus starting the lifecycle once again (Mathews, 2005; Lee *et al.*, 2007) (Figure 1.1).

HOST MICROENVIRONMENTS

As the parasite moves through its life cycle, it will encounter an array of different microenvironments, including the blood, CSF, midgut, hemolymph, and salivary glands. Each of these microenvironments varies in its nutrient composition. For example, the fatty acid concentration in CSF is only 0.2% of that in the bloodstream. This is important because the parasite has an enormous

fatty acid requirement. Fatty acids are the structural components of membranes, signaling molecules, and most importantly, they are a part of the glycosylphosphatidylinositol (GPI) anchors, which are important for anchoring surface molecules involved in immune evasion and nutrient acquisition. Variant surface glycoproteins (VSGs) are among the GPI anchored proteins on the surface of *T. brucei*. VSG molecules mediate antigenic variation, which is the major immune evasion tactic employed by *T. brucei*. Each GPI anchor, which number more than 10^7 (covering the entire surface of the parasite), is myristoylated; two myristates are connected to each GPI anchor. Myristate is a relatively scarce fatty acid in the bloodstream (Paul *et al.*, 2001; Vigueira and Paul, 2011). Since fatty acids are in such high demand and the parasite persists in environments that are nearly devoid of fatty acids, the parasite can compensate for this lack of host fatty acids by synthesizing fatty acids *de novo*. Understanding the interplay between fatty acid synthesis and fatty acid uptake will be important to determine how the parasite meets its fatty acid requirements in challenging environments. There is nothing known about the relative importance of fatty acid synthesis versus fatty acid uptake *in vivo*.

FATTY ACID SYNTHESIS

Fatty acid synthesis (FAS) is a cyclical process involving four enzymatic reactions that extend an acyl chain by two carbons with each successive turn of the cycle. The first reaction is a condensation reaction of the acyl chain with a malonyl group, the two carbon donor molecule, a reaction catalyzed by a

ketoacyl synthase. The resulting ketoacyl intermediate is reduced by a ketoacyl reductase, dehydrated by a dehydratase, and finally reduced by enoyl reductase, to yield a product fatty acyl chain that is two carbons longer (Lee *et al.*, 2007) (Fig. 1.2). FAS pathways implement the same four basic reactions; however, there are different FAS pathways that differ on many other levels.

There are two basic types of FAS pathways: eukaryotic type I and prokaryotic-origin type II. The eukaryotic type I FAS utilizes one or two very large multifunctional proteins, whereas the prokaryotic-origin type II FAS implements four distinct proteins, with discrete enzymatic functions (Schweizer and Hofmann, 2004; Smith *et al.*, 2003). There are many eukaryotic organisms that possess both type I and type II FAS pathways. Generally, the type II FAS pathway is localized to organelles that are prokaryotic in origin, i.e. the mitochondria, apicoplasts, and chloroplasts (Goodman *et al.*, 2007; Lee *et al.*, 2007; Stephens *et al.*, 2007). Additionally, some eukaryotic organisms utilize a microsomal elongase (ELO) pathway, complementing their FAS machinery. ELO pathways utilize the same cycle of enzymatic reactions that type I and type II FAS pathways share, but typically only extend long chain fatty acids (16 to 18 carbons) to very long chain fatty acids (greater than 20 carbons in length) (Toke *et al.*, 1996; Oh *et al.*, 1997; Moon *et al.*, 2001; Jakobson, 2006). ELO pathways also differ in other characteristics: they are associated with organellar membranes and utilize coenzyme-A (CoA) rather than an acyl carrier protein as the carrier molecule for acyl intermediates.

***T. brucei* Fatty Acid Synthesis**

When the *T. brucei* genome was completed, it was clear that *T. brucei* lacks a eukaryotic type I FAS pathway (Berriman *et al.*, 2005). Instead, *T. brucei* possesses a cytosolic ELO pathway and a type II mitochondrial FAS pathway. The ELO pathway accounts for the majority (~90%) of *de novo* fatty acid synthesis in the parasite (Lee *et al.*, 2006). There are four ELO genes, which encode the ketoacyl-CoA synthases of the ELO pathway. They have specific but overlapping chain lengths: ELO1 (C4:0-C10:0), ELO2 (C10:0-C14:0), ELO3 (C14:0-C18:0), and ELO4, which is specific for unsaturated long chain fatty acids. The individual ELOs are not essential *in vivo* due to the overlap in substrate ranges. However, it was determined that *T. brucei* exhibits an environmentally dependent and stage specific requirement for its ELO pathway. A conditional knockout of enoyl-CoA reductase (EnCR), an enzyme downstream of all of the ELOs, caused a growth phenotype only when PFs were grown in low lipid media (Lee *et al.*, 2006). However, in BFs, EnCR was essential for growth in normal media and *in vivo* (Soo Hee Lee, personal communication). This suggests that the ELO pathway, as a whole, is essential in *T. brucei*.

The ELO pathway provides an efficient mechanism of FAS in *T. brucei*. The parasite is able to modulate specific ELOs to preferentially produce specific chain length fatty acids, allowing *T. brucei* the flexibility to reside in many different environmental conditions and meet its demands for fatty acids (Lee *et*

al., 2007). Further, the need for a dedicated FAS pathway and subsequent elongation pathway is eliminated, since the ELO pathway serves both purposes.

In addition to its ELO pathway, the parasite does possess a mitochondrial type II FAS pathway. It is responsible for ~10% of the total fatty acids produced in the parasite and its primary products are C8:0 and C16:0. C8:0 is a precursor for lipoic acid, which is an important prosthetic group for many mitochondrial enzymes (Lee *et al.*, 2007; Stephens *et al.*, 2007). The mitochondrial pathway is also essential for parasite growth. In both PFs and BFs, reduction of the acyl carrier protein (ACP) by RNAi or conditional knockout resulted in reduced mitochondrial FAS and slowed parasite growth in culture (Stephens *et al.*, 2007).

Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step of fatty acid synthesis: the conversion of acetyl-CoA to malonyl-CoA, the two-carbon donor (Barber *et al.*, 2005). ACC is part of the biotin carboxylase family, which possesses a biotin prosthetic group that transfers CO₂ groups from bicarbonate to their acceptors (e.g. acetyl-CoA).

T. brucei ACC (TbACC) was recently characterized and was found to be similar to the ACCs found in other eukaryotes, but it contains a truncated N-terminus (Vigueira and Paul, 2011). The truncated N-terminus lacks the conserved serine residue found in human ACC1 and ACC2. This serine is the site of post-translational regulation by AMP-activated protein kinase (AMPK) (Barber *et al.*, 2005).

TbACC is localized to the cytosol in distinct puncta. The punctate pattern did not localize to other punctate organelles known in *T. brucei*, i.e. lipid droplets and glycosomes. TbACC is required for fatty acid elongation, and RNAi of TbACC reduced ELO activity. ACC is dispensable for growth in normal media for both BFs and PFs. In contrast, when PFs were grown in media containing ~20% of the serum lipids compared to normal media, there was a significant reduction in growth of the parasites. This reduction can be surmounted by the addition of fatty acids to the media. BFs grown in low lipid media showed no effect. However, it was determined that ACC is required for full virulence in a mouse model. When ACC-RNAi was induced *in vivo*, the mouse lived ~11 days longer than mice infected non-uninduced ACC parasites (Vigueira and Paul, 2011).

In Chapters 2 and 3, I explore two possible explanations as to why ACC is dispensable in culture but required *in vivo*. Chapter 2 examines ACC's role in immune evasion, specifically the upregulation of endocytosis to clear surface immunomodulatory molecules, while Chapter 3 examines ACC's role in host adaption, specifically the conservation and maintenance of internal lipid stores in media containing differential lipid content. These studies significantly broaden our understanding of ACC and serve to connect FAS to immune evasion and nutrient storage.

***T. brucei* IMMUNE EVASION**

T. brucei is famous for its ability to evade the mammalian immune system via antigenic variation. BFs are covered in a dense coat of 10^7 identical variant

surface glycoproteins (VSGs). *T. brucei* is able to mediate VSG switching within the parasite population by a hierarchical regime of expression of more than 1600 different VSG genes (Barry and McColloch, 2001; Donelson, 2003; Pays *et al.*, 2004; Pays, 2006). Before the parasite is transmitted to the mammalian host, the metacyclic form found in the insect salivary glands is primed for antigenic variation. It displays a metacyclic specific protein coat, metacyclic variant antigen type 4 (MVAT). The parasite then switches to a VSG coat upon transmittance into the mammalian host. The parasites will continue to switch their VSG coats throughout the course of infection, until host death (Field and Carrington, 2009).

VSGs cover almost the entire surface of the parasite (~95%), except for a small area known as the flagellar pocket (~5%). VSGs are embedded into the membrane via a GPI anchor. The VSG-GPI anchor is myristoylated. Myristate is a scarce fatty acid in the mammalian host bloodstream, and $\sim 2 \times 10^7$ myristates are needed for the VSG coat. This creates an astounding need for fatty acids (especially myristate) and likely a reliance on FAS in addition to uptake (Paul and Vigueira, 2011).

T. brucei also employs a second immune evasion tactic: the upregulation of endocytosis to clear surface immunomodulatory molecules. Endocytosis rates are extremely high in BFs, ~10fold higher than PFs (Natesan *et al.*, 2007). The general upregulation of endocytosis is believed to be involved in the evasion of the innate immune system (Field and Carrington, 2009). Upregulation of fluid phase and receptor-mediated endocytosis effectively clears surface bound

antibodies and complement (Barry, 1979; Ferrante and Allison, 1983; Pal, *et al.*, 2003; Engstler *et al.*, 2007). Fluid phase endocytosis can only occur at the flagellar pocket (Overath and Engstler, 2004). Despite this restricted space, *T. brucei* achieves enormously high membrane turnover rates: i.e. complete VSG turnover occurs in ~12 minutes (Engstler *et al.*, 2004).

In *T. brucei*, fluid phase endocytosis occurs at the flagellar pocket and begins with an invagination of the membrane. The fluid cargo is directed to an early endosome, which becomes a more acidic late endosome. The late endosome fuses with a lysosome, where cargo is either degraded or further transported within the cell (Field and Carrington, 2009).

Receptor-mediated endocytosis occurs when a receptor such as the transferrin receptor binds transferrin. The receptor and ligand are then engulfed into a short term endocytic compartment, known as a recycling endosome. The recycling endosome sorts out the transferrin necessary for iron acquisition and quickly recycles the GPI-anchored transferrin receptor back to the surface to restart the entire process. *T. brucei* uses a similar pathway to clear antibodies and complement from its surface via VSG recycling. VSG is pulled into the flagellar pocket with its bound antibodies/complement, engulfed into a VSG recycling endosome where proteases selectively degrade the antibodies, and the unbound VSG is recycled back to the surface (Kabiri and Steverding, 2000; Chung *et al.*, 2004; Chung *et al.*, 2008; Field and Carrington, 2009).

In Chapter 2, I determine the role of ACC and FAS in both fluid phase and receptor-mediated endocytic pathways. I also look at the clearance of surface immune molecules. First, I examined the efficiency at which ACC-RNAi mutants can clear surface bound antibodies; then, I determined the efficiency in which they are able to avoid complement mediated lysis. These studies have extended our knowledge about the interconnection between ACC/FAS, endocytosis, and immune evasion.

EXTRACELLULAR LIPID UPTAKE

Lipid uptake has been extensively studied in the mammalian system and varies greatly dependent upon specific tissue and/or cell type. In this section, I will summarize the primary lipid acquisition mechanisms.

Endocytosis of lipoproteins is one of the primary routes of lipid uptake. Lipoproteins consist of a polar surface mono-layer, containing phospholipids, apolipoproteins, and cholesterol, and a non-polar inner core, containing triglycerides and cholesterol esters (Wasan *et al.*, 2008). Once endocytosed, the lipoprotein is transported to the lysosome, where the components are liberated. Additionally, specific phospholipids can be acquired from donor lipoprotein particles and inserted directly into the membrane (Engelmann *et al.*, 2010).

Fatty acid uptake is a second major mechanism of lipid acquisition. Fatty acids have very low aqueous solubility and are therefore usually associated with membranes or proteins. This is typically albumin in the mammalian bloodstream. Fatty acids must first dissociate from albumin and partition into the outer leaflet of

the phospholipid bilayer to become internalized. Next, a “flip-flop” must occur, where the orientation of the fatty acid head group flips from the outer to the inner lipid-water interface. Once this occurs, the fatty acid can be bound, activated, or incorporated into more complex lipid species (Glatz *et al.*, 2010).

Fatty acid uptake can occur by protein-mediated active transport and by passive diffusion (Glatz *et al.*, 2010). Fatty acid transport protein 1 (FATP1) is localized to the cellular membrane and actively moves fatty acids into the cell. Other proteins are involved in fatty acid uptake by facilitating diffusion. CD36 and the peripheral membrane fatty acid binding protein function in this manner, binding fatty acids and concentrating them on the membrane surface. The bound fatty acids are more easily partitioned into the membrane than albumin-associated fatty acids; therefore, uptake rates are greatly increased.

Lipid rafts may play a role in fatty acid uptake as well. Caveolin exhibits high-affinity fatty acid binding. Its main role is to provide a plasma membrane docking site for CD36, which is predominantly localized to caveolae, but is dispersed throughout the plasma membrane when lipid rafts are disrupted by cholesterol depletion. There is a decrease in fatty acid uptake when CD36 is mislocalized (Ehehalt *et al.*, 2006; Glatz *et al.*, 2010).

Other proteins that are not associated with the plasma membrane may also play a role in fatty acid uptake. Fatty acids must first be activated before they can be broken down for energy or incorporated into a more complex lipid species. The activation process involves esterification to CoA by a family of

enzymes called acyl-CoA synthetases (ACS). Internalized fatty acids can leave the cell through diffusion if this activation process does not occur. Once esterified to the CoA, the resulting acyl-CoA is membrane impermeant. Thus, ACS increases the retention of fatty acids brought in by either passive diffusion or protein-mediated active transport (Milger *et al.*, 2006).

Lipid Uptake in *T. brucei*

There are a few mechanisms of lipid acquisition that have been described in *T. brucei*. First, BFs can uptake both low and high density lipoproteins via endocytosis (Coppens *et al.*, 1995; Green *et al.*, 2003). They also possess a lipoprotein scavenger receptor that compliments this process (Green *et al.*, 2003; Thomson *et al.*, 2009). PFs uptake lipophorin from the tse tse hemolymph, presumably via a similar mechanism to BF lipoprotein uptake. The uptake of lipophorin has been demonstrated in the related parasite, *T. rangeli* (Folly *et al.*, 2003) and a malarial parasite, *Plasmodium gallinaceum* (Atella *et al.*, 2009). Additionally, *T. brucei* has a specialized mechanism for the acquisition of phospholipids involving the coordinated activity of three proteins; this mechanism was validated *in vitro* for the uptake of lysophosphatidylcholine (Bowes *et al.*, 1993).

There is little known about fatty acid uptake in *T. brucei*. None of the proteins involved specifically in uptake have been identified or characterized. There is evidence that *T. brucei* may employ the same two general mechanisms that are found in human cells, i.e. protein-mediated active transport and passive

diffusion. There are five ACSs in the genome and some early characterization has been completed for them (Jiang *et al.*, 2000; Jiang *et al.*, 2001; Jiang *et al.*, 2004). In this thesis, I delve lightly into fatty acid uptake, utilizing both fluorescently labeled and unlabeled fatty acids. While I did not attempt to characterize proteins or methods of uptake, I did elucidate the downstream pathway, i.e. where fatty acids go after their entrance into the cell in Chapter 3.

LIPID DROPLETS

Lipid droplets are highly dynamic lipid storage organelles found mainly in eukaryotic organisms and some prokaryotes. Lipid droplets are usually composed of neutral lipids. Triacylglycerols and cholesterol esters are the major storage lipid, though diacylglycerols and fatty acids have also been identified within the organelles (Goodman, 2009; Reue, 2011). Surrounding the neutral lipid storage core, there is a phospholipid monolayer, with similar composition to the membrane found in the ER, with the acyl chains facing inward. Proteins involved in membrane trafficking, such as the Rab family and Arf-1 localize to the lipid droplet surface and are believed to play direct roles in the cells' endocytic and exocytic processes (Fig. 3)(Goodman, 2009). Additionally, there is proteomic data localizing peroxisomal, mitochondrial, and ER-specific proteins to the surface of lipid droplets, increasing the evidence that these organelles are integrally involved in trafficking between organelles (Goodman, 2008). Aside from trafficking, lipid droplets function in energy storage, lipid metabolism, and protection against the toxic effects of non-esterified sterols, lipids, and

exogenous non-polar compounds. However, the extent of involvement in each of these roles is highly variable depending upon species, cell, and tissue type (Flaspohler *et al.*, 2010).

Lipid droplets are dynamic organelles. They are able to self-associate and associate with other organelles, such as endosomes and ER. The regulation of lipid droplet size and number is largely undefined. It is known that certain environmental conditions can influence both size and number, e.g. an increase in extracellular lipid content results in an increase in lipid droplet number and/or size (Reue, 2011; Flaspohler *et al.*, 2010). Additionally, it is not known exactly how lipid droplets form. There are two main hypotheses on lipid droplet formation. The first hypothesis claims that lipid droplets form as buds from the ER, an idea supported by proteomic data that determined that the same proteins found on the surface of lipid droplets are found on the surface of the ER (Goodman, 2008). Also, 96% of lipid droplets were scored as associating with ER in yeast (Goodman, 2009). Lastly, there is some evidence in yeast that lipid droplets may not leave the ER. Instead of becoming independent organelles, they remain associated with the ER and could be considered specialized parts of the ER (Goodman, 2008).

The alternative hypothesis of lipid droplet formation centers around the idea that lipid droplets are not buds of the ER. Rather, they are simple organelles that are manufactured separately from the ER and are able to associate with many organelles within the cell as needed. This hypothesis garners support

because lipid droplets do not just associate exclusively with the ER but associate with many different organelles within the cell. For example, within a muscle cell, the lipid droplets also associate with mitochondria, and it is believed that this association enhances energy production by coupling the release of fatty acids with their beta oxidation (Goodman, 2009). Lipid droplets are also known to associate with peroxisomes in both animals and plants (Goodman, 2008; Goodman, 2009). In plants, lipid droplets supply the phospholipid membranes of glyoxysomes. In animal cells, lipid droplets interact with peroxisomes via what is believed to be bidirectional lipid movement between organelles, because the lipid droplet core is rich with ether lipids that are synthesized within the peroxisomes. Lastly, lipid droplets associate with endosomes (Goodman, 2009). High exogenous concentrations of cholesterol and fatty acids increase caveolin on the surface of lipid droplets and a decrease their appearance on the ER, suggesting that uptake into endosomes will directly traffic these lipids to lipid droplets, bypassing the ER (Goodman, 2008; Goodman, 2009).

Lipid Droplets in *T. brucei*

Lipid droplet synthesis and characterization in *T. brucei* is a largely unexplored area of research. It is unknown how lipid droplets are synthesized and trafficked within the parasite. Additionally, the proteins that are involved in these processes or colocalize to the surface of lipid droplets are mostly unknown. Recently, one kinase, Lipid Droplet Kinase (LDK), was found to localize to the surface of lipid droplets in *T. brucei*. LDK is on or embedded within the

phospholipid monolayer of lipid droplets and is involved in both the synthesis and maintenance of lipid droplets (Flaspohler, *et al.*, 2010). When LDK was knocked down by RNAi, lipid droplet number decreased. Further, when uninduced cells were exposed to low lipid media for 24 hours, there was an increase in lipid droplet formation, presumably due to the upregulation of fatty acid synthesis. However, LDK-RNAi cells did not experience an increase in lipid droplet number when subjected to the same treatment, indicating a role in both lipid droplet synthesis and maintenance in *T. brucei* (Flaspohler, *et al.*, 2010).

In Chapter 3, I characterized *T. brucei* lipid droplet maintenance and synthesis further. I examined the effects of exogenous lipid content on lipid droplet number and maintenance. I then looked at the role of fatty acid synthesis, specifically through the knockdown of ACC by RNAi, on lipid droplet number and maintenance. Lastly, I determined the effects of both ACC-RNAi and the environmental status of lipids on bulk neutral lipid content in *T. brucei*. My studies represent an initial characterization of lipid droplet biology in *T. brucei*.

FIGURES

Figure 1.1: *T. brucei* life cycle. *T. brucei* alternates between its insect host, the tse tse fly, and its mammalian hosts. Figure adapted from Lee S. H., Stephens J. L., and Englund P. T. (2007) A fatty-acid synthesis mechanism specialized for parasitism. My research focuses on the Procyclic form (PF) and “Long, slender” form (BF) *Nat Rev Microbiol* **5**: 287-297.

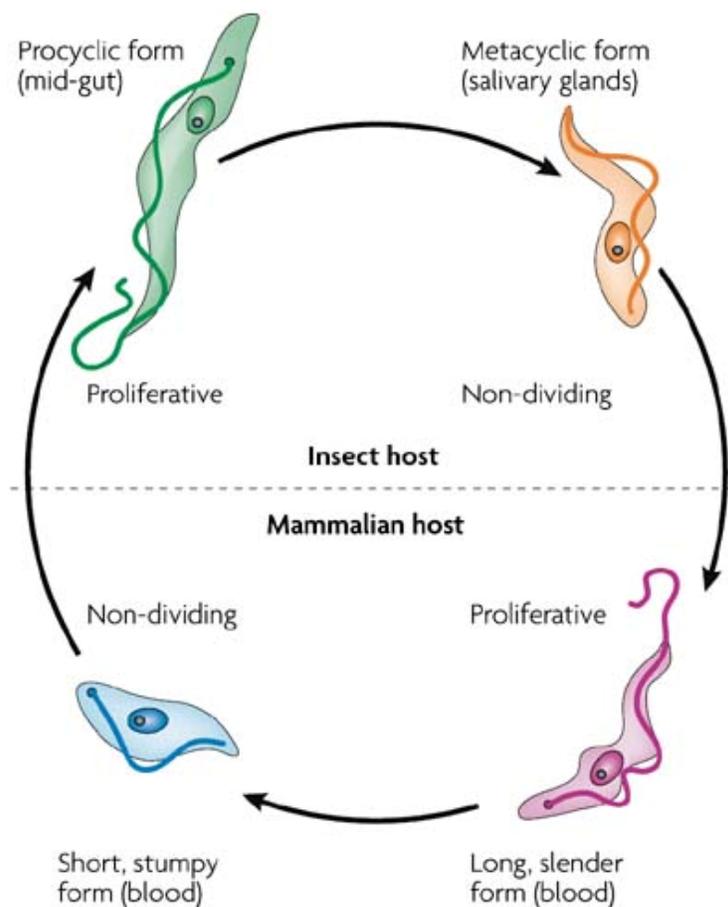


Figure 1.2: Enzymatic reactions of the fatty acid synthesis elongase pathway by *T. brucei*. The cycle consists of four enzymatic reactions. Each turn of the cycle yields an acyl-CoA product that is 2 carbons longer. Figure adapted from Lee S. H., Stephens J. L., and Englund P. T. (2007) A fatty-acid synthesis mechanism specialized for parasitism. *Nat Rev Microbiol* **5**: 287-297.

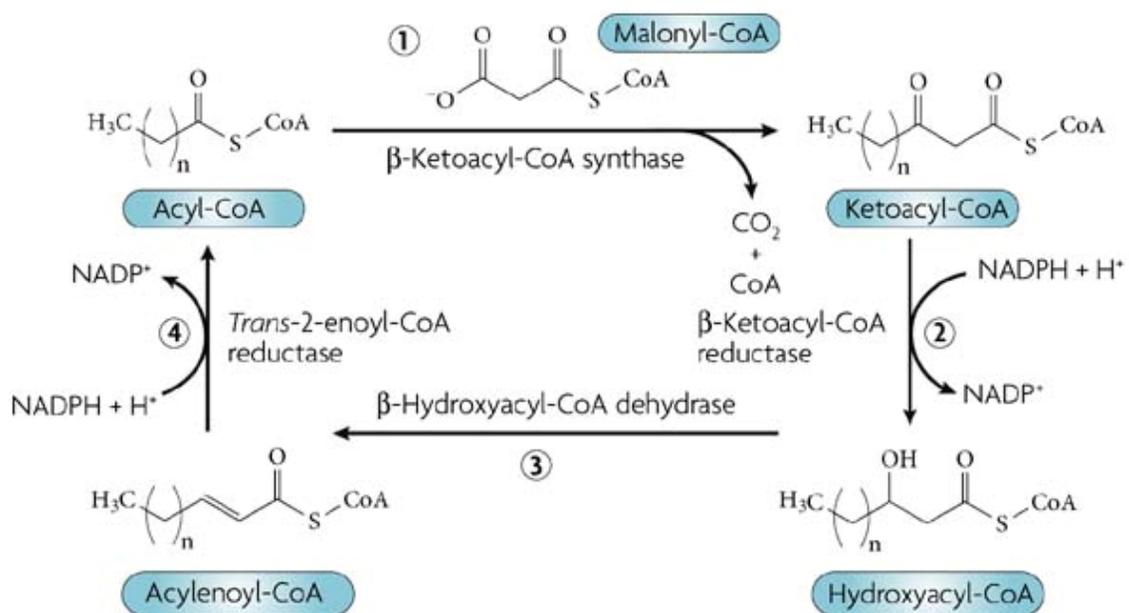
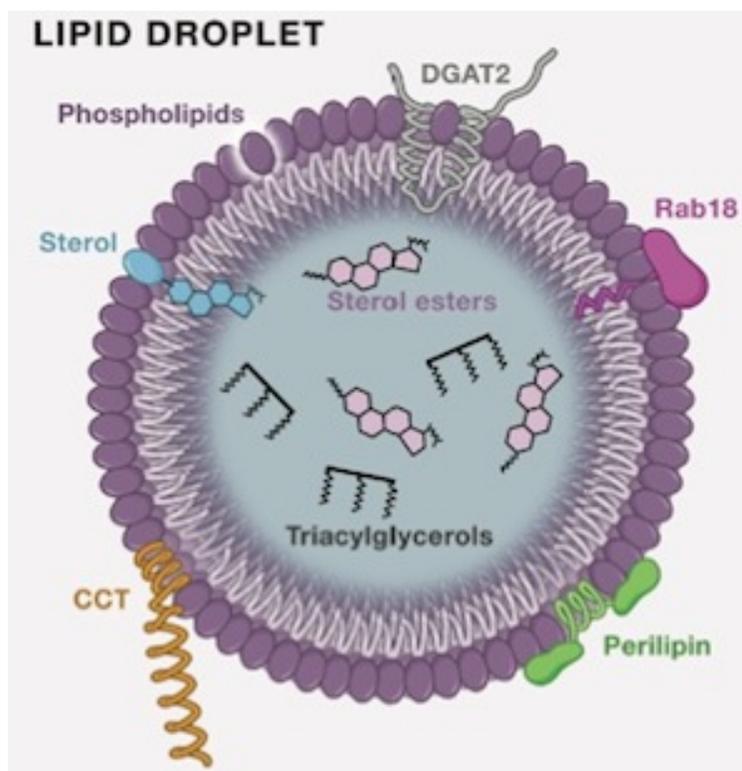


Figure 1.3: Lipid Droplet. Lipid droplets are surrounded by a phospholipid monolayer and contain a neutral lipid storage core. Figure adapted from Kraemer N., Guo, Y., Farese, R., and Walther, T. (2009) Snapshot: Lipid Droplets. *Cell* **139**:5,1034.



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THE ROLE OF ACETYL-COA CARBOXYLASE IN *T. brucei* IMMUNE EVASION

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ABSTRACT

Trypanosoma brucei, the causative agent of human African trypanosomiasis, employs the upregulation of endocytosis to clear lytic cell surface immune complexes as an immune evasion tactic. This strategy involves membrane turnover, which presumably requires fatty acid synthesis (FAS) and may depend on environmental lipid availability. To explore the role of fatty acid synthesis in membrane turnover, we have examined the consequences of RNA interference (RNAi)-mediated reduction of acetyl-CoA carboxylase (ACC), the first committed step of fatty acid synthesis. First, we examined both fluid phase endocytosis, using a fluorescent dextran marker, and receptor-mediated endocytosis, using a fluorescent transferrin marker, in bloodstream form (BF) ACC-RNAi parasites and found a 54-88% reduction in both endocytic pathways in low, normal, and high lipid media. Next, we tested the effect of ACC-RNAi on the parasites' ability to clear surface-bound antibodies and determined that the ACC-RNAi cells required more time (25-27%) to clear fluorescently conjugated antibodies from the surface. Lastly, we tested the effect of ACC-RNAi on complement mediated lysis and found that ACC-RNAi resulted in a 31-45%

reduction in the ability to avoid complement-mediated lysis. Therefore, *T. brucei* relies on ACC to maintain high levels of endocytosis, clear immune complexes, and avoid complement mediated lysis, suggesting that ongoing fatty acid synthesis is required for immune evasion in the mammalian host

INTRODUCTION

Trypanosoma brucei, the causative agent of human African trypanosomiasis (HAT) and nagana in livestock, is a protozoan parasite vectored by the tse tse fly that infects both humans and livestock. Over the course of infection, the parasite encounters a diverse array of microenvironments, including the bloodstream and cerebrospinal fluid, which differ in nutrient content. Notably, there is a ~300X lower concentration of lipids in the cerebrospinal fluid compared to the bloodstream (Lentner, 1981). *T. brucei* has an obligate fatty acid requirement because they are important constituents of the glycosylphosphatidylinositol (GPI) anchors, which anchor cell surface glycoproteins. Bloodstream form (BF) *T. brucei* is covered with 1×10^7 GPI-anchored variant surface glycoproteins (VSGs), each requiring two myristates (Paul *et al.*, 2001). To meet this requirement, the parasite is able to acquire fatty acids in two ways: *de novo* synthesis of fatty acids or uptake of fatty acids from the host (Smith and Bütikofer, 2010). Fatty acid uptake requires less energy than synthesis. However, because the abundance in some environments is very low (i.e. the cerebrospinal fluid) the parasite is unable to rely solely on uptake to

satisfy its need. Therefore, *de novo* synthesis is utilized to meet the parasite's fatty acid requirement.

Two fatty acid synthesis pathways have been described in *T. brucei*. The fatty acid elongase (ELO) pathway of the endoplasmic reticulum serves as the major pathway for synthesis, accounting for ~90% of fatty acids synthesized (Lee *et al.*, 2006). The second pathway is found in the mitochondrion and accounts for ~10% of the fatty acids synthesized (Stephens *et al.*, 2007; Guler *et al.*, 2008). Both pathways are essential (Lee *et al.*, 2006; Stephens *et al.*, 2007). The ELO pathway is initiated by the conversion of acetyl-CoA to malonyl-CoA via acetyl-CoA carboxylase (ACC). This is considered the first committed step of fatty acid synthesis (Tehlivets *et al.*, 2007; Saggerson, 2008). Previously RNA interference (RNAi) of *T. brucei* ACC, demonstrated that in BFs, TbACC was not required for growth in culture but was necessary for full virulence in mice (Vigueira and Paul, 2011). One major difference between growth in culture and growth in mice is the presence of the host immune system. Thus, we propose that ACC is required in *T. brucei* for immune evasion.

The major immune evasion tactic of *T. brucei* is antigenic variation. The BF parasite is covered in a dense coat of 10^7 VSGs and is able to mediate coat switching within the population following a hierarchal regime of expression of ~1000 different VSG genes (Barry and McCulloch, 2001; Donelson, 2003; Pays *et al.*, 2004; Pays, 2006). The parasite switches from an initial coat, metacyclic variant antigen type 4, expressed in the metacyclic stage in the salivary glands,

to a single VSG, which continues to switch throughout the course of infection until the death of the host (Field and Carrington, 2009).

A second immune evasion tactic mechanism involves the upregulation of endocytosis. Endocytosis rates in BFs are ~10 fold higher than those of the insect procyclic form (PF) parasites (Natesan, *et al.*, 2007). Upregulation of fluid phase endocytosis and receptor mediated endocytosis can effectively clear antibodies and serum complement bound to the parasite surface (Balber *et al.*, 1979; Ferrante and Allison, 1983; Donelson, *et al.*, 1998). It is believed that this upregulation is one of the early tactics used to evade the innate immune system (Field and Carrington, 2009). Endocytosis occurs at the flagellar pocket, which accounts for only ~5% of the entire membrane (Morgan *et al.*, 2002a,b; Overath and Engstler, 2004). Antibody clearance is achieved by the internalization of VSGs through the flagellar pocket into a recycling endocytic pathway, where the VSG-antibody complex is sorted, with the antibody being degraded and released outside the cell and the VSG recycled back to the surface (Donelson *et al.*, 2009; Field and Carrington, 2009). The avoidance of complement-mediated lysis is also attributed, in part, to the upregulation of endocytosis. When antibodies bind the surface of *T. brucei*, complement is recruited to lyse the cells (Balber *et al.*, 1979; Russo *et al.*, 1993, Donelson *et al.*, 1998; O'Beirne *et al.*, 1998; Engstler *et al.*, 2007; Natesan *et al.*, 2007). Likely, *T. brucei* is able to evade this mechanism by internalizing antibody-bound VSGs and recycling unbound VSGs back to the surface.

Here, we hypothesize that ACC is required for full virulence in mice because fatty acid synthesis is important in maintaining elevated endocytosis levels. Because *T. brucei* encounters microenvironments differing in lipid content and possesses the ability to synthesize fatty acids *de novo* and take up host fatty acids, we explore the requirement for ACC in the upregulation of endocytosis and the clearance of surface-bound antibody and complement. We show that ACC-RNAi reduces both fluid phase and receptor-mediated endocytosis. We also determine that ACC-RNAi reduces the parasite's ability to clear surface-bound antibodies. Finally, we demonstrate that ACC is necessary to avoid complement-mediated lysis.

RESULTS

ACC-RNAi reduces fluid phase and receptor-mediated endocytosis

As shown previously, while ACC-RNAi did not affect the growth of *T. brucei in vitro*, it did affect the virulence of *T. brucei* within the mouse (Vigueira and Paul, 2011). To investigate this decrease in virulence, we decided to investigate one of the parasite's immune evasion tactics, increased endocytosis. BF parasites exhibit a very high rate of endocytosis, which is proposed to help BFs evade the immune system (Field and Carrington, 2009). Because this increase in endocytosis is related to host adaption and immune evasion (Engstler and Overath, 2004; Field and Carrington, 2009), we assessed the parasites' ability to undergo both fluid-phase and receptor mediated endocytosis in BF ACC-RNAi cells. Further, because the parasite can encounter both low and high

lipid conditions in the host, we assessed the effect of ACC-RNAi on these processes under three different lipid conditions: “normal” lipid (12% serum, which corresponds to the standard culture media), “low” lipid (4% serum), and “high” lipid (22% serum).

ACC-RNAi effectively reduces detectable ACC protein levels in BFs (96% compared to uninduced cells) (Fig. 2.1A), under all serum lipid levels tested. This reduction has no impact on the doubling time, even when grown in low lipid medium (Fig. 2.1B). After 9 days of ACC-RNAi induction, endocytosis of FITC-dextran was reduced by $88 \pm 8\%$ when *T. brucei* was grown in normal lipid media (Fig. 2.2B). ACC-RNAi induction (9 days) also reduced the amount of FITC-dextran endocytosed by *T. brucei* by $54 \pm 22\%$ in low lipid media (Fig. 2.2C) and $55 \pm 23\%$ in high lipid media (Fig. 2.2D). Additionally, when cells were grown in low lipid media for 3 or more days, there was a 3-4 fold reduction in fluid-phase endocytosis compared to normal and high lipid media, regardless of ACC-RNAi induction (Fig. 2.2B-D).

Next, we assessed the effect of ACC-RNAi on transferrin receptor-mediated endocytosis, because its endocytic pathway is similar to the VSG recycling pathway (Steverding, *et al.*, 1995; Overath and Engstler, 2004; Subramanya *et al.*, 2009). After 9 days of induction, ACC-RNAi decreased endocytosis of Alexa Fluor-conjugated transferrin by $87 \pm 14\%$ in normal lipid media (Fig. 2.3B), $73 \pm 19\%$ in low lipid media (Fig. 2.3C), and $61 \pm 8\%$ in high

lipid media (Fig. 2.3D). Unlike for FITC-dextran uptake, we observed no effect of media serum lipids on transferrin receptor-mediated endocytosis.

ACC-RNAi reduces clearance of surface-bound antibodies

Previously, it was demonstrated that the increased endocytosis rates in BFs serves as a mechanism to clear surface antibody complexes (Steverding, et al., 1995; Overath and Engstler, 2004; Field and Carrington, 2009). This clearance process is believed to aid the parasites' survival during early stages of infection (Field and Carrington, 2009). Since we found a decrease in both fluid phase and receptor mediated endocytosis upon ACC-RNAi, we assessed the ability of *T. brucei* to clear surface-bound antibody complexes using a previously published assay (Pal *et al.*, 2003). First, we isolated a population of BF ACC-RNAi cells expressing the 221 VSG variant by fluorescence-activated cell sorting. We then incubated these 221 VSG-expressing cells with a polyclonal anti-221 VSG antibody and tracked its endocytosis and subsequent recycling to the surface via a fluorescently-labeled secondary antibody. Silencing ACC for 9 days led to an increase in the time it took for the parasites to endocytose and recycle their VSG back to the surface (Fig. 2.4A-D; Table 1). The increase in recycling time (Δt) was similar in all lipid conditions, with a $25 \pm 8\%$, $26 \pm 8\%$, and $27 \pm 9\%$ reduction in low, normal, and high lipid media, respectively (Fig. 2.4; Table 1).

ACC-RNAi reduces the avoidance of complement-mediated lysis

T. brucei also avoids complement-mediated lysis (Balber *et al.*, 1979; Donelson *et al.*, 1998) through the endocytosis and degradation of surface bound

VSG-antibodies. Because BF ACC-RNAi parasites have a defect in endocytosis and the ability to clear surface bound antibodies, we explored whether ACC-deficient parasites were also impaired in their ability to avoid complement-mediated lysis. To do this, we incubated 221 VSG-expressing BF ACC-RNAi cells with anti-221 VSG polyclonal antibody and guinea pig complement and monitored cell viability using the Cell Titer Blue assay. Induction of ACC-RNAi for 9 days resulted in a 31-45% increase cell sensitivity to complement-mediated lysis in all three lipid medias (Fig. 2.5). Lysis was confirmed microscopically (Data not shown).

DISCUSSION

Previously, it was demonstrated that ACC was necessary for full virulence in the mouse model (Vigueira and Paul, 2011). Here, we investigate the role of fatty acid synthesis by ACC-RNAi in one of the major immune evasion mechanisms employed by *T. brucei*, the upregulation of endocytosis to clear surface immune complexes and to avoid complement mediated lysis. We demonstrate that there is a reduction in both fluid phase and receptor-mediated endocytosis in ACC-RNAi parasites. Further, this reduction in endocytic activity increases the kinetics of VSG-antibody complex clearance from the surface of ACC-RNAi BFs. Lastly, because antibodies are not cleared as quickly from the surface of ACC-RNAi parasites, they are more sensitive to complement-mediated lysis.

The reduction in both fluid phase and receptor-mediated endocytosis is believed to be due to the parasite's inability to undergo membrane turnover. As membrane is depleted by endocytic processes, the parasite cannot maintain the high levels of membrane turnover necessary to continue the high rates of endocytosis. Since fatty acids are constituents of membrane lipids, the depletion of fatty acids must affect the parasite's ability to synthesize additional membrane lipids. Therefore, the decrease in both fluid phase and receptor-mediated endocytosis can be attributed to a downstream effect of the loss of fatty acid synthesis, which in turn results in a lack of the basic building blocks of plasma membrane lipids. Additionally, lipids play a role in secondary signaling in endocytic pathways. For example, diacylglycerol is a secondary signaling molecule released from phospholipids by phospholipase C (Davis *et al.*, 1985). Together, the inability to replenish the membrane and signaling lipids could provide an explanation as to why ACC-RNAi negatively affects endocytosis.

One interesting finding is that there was a 3-4 fold reduction in the fluid phase endocytic pathway, regardless of induction state, in parasites grown in low lipid medium for 3 or more days. However, this general reduction in endocytic capabilities was not observed in the same parasites when assaying for receptor mediated endocytosis. The dichotomy between the effect of ACC-RNAi on these two pathways cannot be attributed to vesicle formation, i.e. fluid phase vesicles are not formed as readily as receptor-mediated. Vesicle formation is the same for

both endocytic processes in *T. brucei* (unlike in mammalian cells). To date, only clathrin coated vesicles have been described in *T. brucei* (Allen *et al.*, 2003).

We could also surmise that fluid phase endocytosis is somehow a more membrane intensive activity than receptor-mediated endocytosis; however, this is unlikely. Both processes occur at the same location and utilize very similar machinery, i.e. clathrin coated vesicles and Rab family proteins (Jeffries *et al.*, 2001; Denny *et al.*, 2001; Pal *et al.*, 2002; Pal *et al.*, 2003; Grunfelder *et al.*, 2003.)

It may be interesting to explore this finding further by growing parasites in low lipid medium for time points shorter than 3 days, where there may be a vast increase in endocytosis, similar to what was observed in yeast (Conner and Schmid, 2003). If there is a large increase in fluid phase but not receptor-mediated endocytosis, perhaps the parasite begins to down regulate fluid phase endocytosis to preserve membrane for essential processes such as receptor-mediated endocytosis of transferrin for iron acquisition.

In addition to effects on endocytosis, ACC-RNAi also reduced the clearance of antibody complexes from the parasite surface. It is important to note that this was attributed to endocytosis and not to migration of VSG to the flagellar pocket nor exocytosis of VSG and antibody back to the surface. When examining the kinetic curves, there is an obvious delay in endocytosis but not in exocytosis back to the surface. This delay could be due to a defect in the migration of VSG to the flagellar pocket or to defects in the VSG recycling pathway. Antibody-

bound VSGs migrate to the flagellar pocket via hydrodynamic flow, with the antibodies bound to the VSGs acting like “sails” driven by parasite movement (Engstler *et al.*, 2007). We observed no defects in parasite movement upon microscopic examination, which argues against a defect in the hydrodynamic flow of VSGs toward the flagellar pocket. However, as problems in membrane composition could reduce the efficiency of the VSG flow without a noticeable change in parasite movement; we further examined antibody-bound VSG migration by microscopy and observed “capping” near the flagellar pocket. This supports our conclusion that the migration remains unaffected, while there is a delay in the endocytosis of the antibody-bound VSGs from the surface. Further, the recycling endocytosis pathway of VSG is similar to that of GPI anchored transferrin receptor (O’Beirne *et al.*, 1998; Field and Carrington, 2009), which we showed was defective under ACC-RNAi conditions. Therefore, we attribute the observed defect in antibody clearance upon ACC-RNAi to a defect in endocytosis rather than in the hydrodynamic flow of VSGs to the flagellar pocket.

Because we demonstrated that ACC-RNAi resulted in a reduction in endocytosis to clear surface bound antibodies, we examined susceptibility to complement-mediated lysis and found that the ACC-RNAi parasites were more sensitive. The increased susceptibility to complement-mediated could be due to decreased endocytosis of surface-bound antibodies. The sensitivity of the ACC-RNAi parasites to complement remained low, even when antibody accumulation of the surface was high. There may be an alternative mechanism involved in the

avoidance of complement-mediated lysis. In *Leishmania spp.*, a closely related trypanosomatid, GP63 is a surface bound protease that aids in its ability to infect cells and avoid complement-mediated lysis by cleaving the complement component C3b to the inactive C3bi (Chang *et al.*, 1986; Liu *et al.*, 1992; Soteriadou *et al.*, 1992; Brittingham *et al.*, 1995; Yao *et al.*, 2003; Olivier and Hassani, 2010). Upon a genome wide search, it was determined that *T. brucei* possesses several genes that encode a similar protein and may be involved in the avoidance of complement-mediated lysis (Donelson *et al.*, 1998; Yao *et al.*, 2003).

We determined a role for ACC in one immune evasion mechanism; however, the major immune evasion mechanism employed by *T. brucei* is not the upregulation of endocytosis, but rather, antigenic variation (Barry and McCulloch, 2001; Donelson, 2003; Pays *et al.*, 2004; Pays, 2006). Future studies should also explore the role of ACC in VSG presentation and switching. As each VSG molecule is GPI anchored to the membrane and each GPI anchor contains 2 myristates (Paul *et al.*, 2001), it is possible that fatty acid synthesis might play a role in both presentation of VSG and switching of VSG. This could explain ACC's dispensability *in vitro* but necessity *in vivo*. We expect that ACC-RNAi parasites would be defective in one or both of these processes due to the high demand of fatty acid synthesis.

Our study examined the effect of ACC-RNAi and reduced fatty acid synthesis in very downstream processes. It is important to note that fatty acids

are important building blocks of most lipids utilized in the cell for membrane biogenesis and signaling. Other groups have examined the effects of phosphatidylinositol phosphate (Bussolino *et al.*, 2001) and sphingolipid synthesis (Conzelmann, 2012) on lipid biogenesis and cell viability and found that both are necessary for lipid biogenesis. Without a source of lipids, the cell ultimately cannot survive. *T. brucei* presents an interesting problem, in that it can uptake and synthesize fatty acids. However, we have demonstrated that uptake alone cannot fulfill the parasite's need. If both fatty acid synthesis and uptake can be blocked, then *T. brucei* would not survive. Continued study of both fatty acid synthesis and fatty acid uptake will be needed to determine the viability of fatty acid metabolism pathways as a potential drug target.

Acknowledgements

We thank Dr. James Morris (Clemson University) for the anti-VSG 221 antibody and Dr. Julie Nelson (University of Georgia) for assistance in isolating cells expressing VSG 221. We thank Drs. Lesly Temesvari, Meredith Morris, and James Morris for helpful discussions. This work was supported by funds from the NIH (R15AI081207-01A1).

MATERIALS AND METHODS

Reagents

All chemicals and reagents used in these studies were purchased from Thermo Fisher Scientific and Sigma except: Iscove's Modified Dulbecco's Medium (IMDM), Minimum Essential Media (MEM), 4'-6-diamidino-2-phenylindole (DAPI), Fluorescein isothiocyanate- dextran (FITC-dextran), transferrin-Alexa Fluor 488, and secondary goat anti-rabbit IgG FITC-conjugated antibody were from Invitrogen; Serum Plus was from JRH Biosciences; and delipidated fetal bovine serum was from Cocalico Biologicals. The AnTAT-221 variant anti-VSG antibody was generated by Drs. David Alexander and Jay Bangs (Univ. of Wisconsin), and was a generous gift from Dr. James Morris (Clemson University).

Trypanosome Cell Lines and Growth Media

BF 90-13 ACC-RNAi cell lines have been described (Vigueira and Paul, 2011) Normal lipid, low, and high lipid growth media were prepared using HMI-9 medium (Hirumi and Hirumi, 1989) supplemented with 2.5 µg/ml G418, 5 µg/ml hygromycin, and 2.5 µg/ml phleomycin. Normal lipid media contained 10% heat-inactivated fetal bovine serum (FBS) and 10% Serum Plus (JRH Biosciences), resulting in a 12% serum lipid content. Low lipid media contained 10% delipidated FBS (Cocalico Biologicals) and 10% Serum Plus, resulting in a 4% serum lipid content. High lipid media contained 20% heat-inactivated FBS and 10% Serum Plus, resulting in a serum lipid content of 22%. ACC-RNAi was

induced by the addition of 1 µg/ml Tetracycline to the media and fresh Tet was added at the time of cell passage every two days. To isolate cells expressing the 221 VSG variant, BF 90-13 ACC-5 lines were incubated with a 1:1000 dilution of anti-221VSG polyclonal antibody (Balber *et al.*, 1979) and 1:100 dilution of polyclonal alexa-fluor 488 conjugated anti-rabbit polyclonal antibody and sorted for 221 VSG expressing cells using the Beckman Coulter MoFlo XDP cell sorter at the Center for Tropical and Emerging Global Diseases Flow Cytometry Center (University of Georgia). This isolated population was used within 15 days of sorting and we observed a ~5% background of switched cells by day 15 post-sorting.

Growth Curves and Streptavidin Blotting

To perform a growth analysis, cultures were back-diluted to $\sim 1 \times 10^4$ cells/ml in fresh media and divided into two. The Induced culture was supplemented with 1 µg/ml Tetracycline to induce ACC-RNAi, while the control uninduced culture received ethanol. The cell densities of the cultures were monitored every day by hemocytometer counts. Average doubling times were calculated from the linear part of the growth curve.

Hypotonic lysates were prepared as described (Morita *et al.*, 2000). Streptavidin blotting can detect the biotin prosthetic group on ACC and was performed essentially as described (Vigueira *et al.*, 2011). Briefly, hypotonic lysates were fractionated on 10% SDS-PAGE gels, transferred to nitrocellulose, and blocked in Wash Buffer (1% dry milk, 1X TBS, 0.05% Tween-20). The blot

was then cut; the top half was probed for ACC with streptavidin-horseradish peroxidase conjugate (SA-HRP) (1:400 in 0.2% dry milk, 1X Tris-buffered saline (TBS), 0.05% Tween-20); and the bottom for tubulin with a mouse anti-tubulin antibody (clone B-5-1-2; Sigma) diluted 1:50,000 in Wash Buffer followed by HRP-conjugated goat anti-mouse IgG antibody (Invitrogen) diluted 1:10,000 in Wash Buffer. Semi-quantitative analysis of the blots was performed using densitometry (NIH Image J software) of exposed films (unsaturated signal within the linear range of the film).

Endocytosis Assays

FITC-dextran and transferrin-Alexa Fluor 488 uptake experiments were performed essentially as described in (Subramanya *et al.*, 2009). BF ACC-RNAi uninduced and induced cells were grown in low, normal, or high lipid media for 3, 6, or 9 days. Cells were harvested by centrifugation (1000 x g) washed once in BBSG (50 mM bicine- Na^+ pH 8, 50 mM NaCl, 5 mM KCl, 70 mM glucose), and resuspended at a density of 5×10^6 cells/mL vol in serum-free HMI-9+1% bovine serum albumin. Cells (5×10^6) were incubated with 2 mg/mL FITC-dextran or 20 $\mu\text{g}/\text{mL}$ transferrin-Alexa Fluor 488 for 10 min at 37 °C. Cells were centrifuged (3000 x g for 5 min at 4°C) and then washed 5 times with ice cold BBSG+2% sodium azide to terminate uptake. Because FITC-dextran fluorescence is sensitive to the lower pH in endocytic organelles, the cells incubated with FITC-dextran were subsequently lysed with 0.2% Triton-X-100 (final concentration) to release all intracellular FITC-dextran. The FITC-dextran incubated cell lysate and

the unlysed transferrin-Alexa Fluor 488 incubated cells were then diluted 1:2 in BBSG + 2% sodium azide and transferred to a 96-well plate, and cell-associated fluorescence was monitored (excitation 490 emission 525) using a FLX800 fluorescence plate reader equipped with KC4 software (BioTek Instruments). Final fluorescence values were obtained by subtracting the “cell only” control from the samples incubated with the fluorescent markers.

Microscopy

Microscopy was performed essentially as described (Field *et al.*, 2004). To monitor endocytosis, $\sim 1 \times 10^7$ BF ACC-RNAi induced and uninduced cells were harvested by centrifugation (800 x g, 10 min), incubated with either 2 mg/ml FITC-dextran or 20 μ g/ml transferrin-Alexa Fluor 488 dissolved in serum-free HMI-9+1% bovine serum albumin 10 min, washed 3 times in ice-cold Voorheis's modified PBS (vPBS) (Nolan *et al.*, 2000), and fixed on ice in 3% paraformaldehyde (w/v) in vPBS for 10 minutes. Fixed parasites were adhered to poly-L-lysine treated glass slides and permeabilized for 10 min in 0.1% Triton X-100 (v/v) in vPBS. The nucleus and kinetoplast were stained with 4'-6-diamidino-2-phenylindole (DAPI) (1 mg/ml in vPBS).

For the antibody clearance studies, VSG 221 expressing ACC-5 cells (induced and uninduced for ACC-RNAi) were grown for 9 days in low, normal, or high lipid media. Cells (1×10^7) were harvested by centrifugation at 800 x g. Cells were washed once with ice cold vPBS and fixed on ice in 3% paraformaldehyde (w/v) in vPBS for 10 min. Fixed parasites were adhered to poly-L-lysine treated

glass slides and incubated in microscopy blocking solution (MBS: 0.5% bovine serum albumin (BSA) (w/v), 5% normal goat serum (v/v), 20% FBS (v/v) in vPBS) for 1 h. The fixed, intact cells were then incubated with 1:1000 rabbit IgG anti-VSG 221 antibody (McDowell *et al.*, 1998) in MBS for 30 min., washed 2X in MBS, then incubated with 20 µg/mL goat anti-rabbit IgG secondary FITC-conjugated antibody (Invitrogen) in MBS for 1 h. The slide was washed 4X in MBS. The nucleus and kinetoplast DNA were stained with DAPI as above.

For timed incubations of internalized antibodies, cells were harvested, washed, then incubated with the primary anti-VSG 221 antibody for various timepoints at 37°C. The cells were immediately washed, fixed in 3% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in vPBS (v/v) prior to incubation with primary and secondary antibodies and DAPI as described above.

Images were collected using a Nikon TE2000 widefield epifluorescence microscope and image acquisition was performed using Nikon NIS Elements software package. Image analysis was further performed using NIH Image J and Adobe Photoshop.

Antibody clearance assay

Antibody clearance assays were performed essentially as described by (Pal, *et al.*, 2003). BF ACC-RNAi uninduced and induced parasites expressing the VSG 221 surface coat were harvested at 1,000 x g and resuspended at 3×10^6 cells/ml in 200 µL of ice cold TES buffer (120 mM NaCl, 5 mM KCl, 3 mM

MgSO₄, 16 mM Na₂HPO₄, 5 mM KH₂PO₄, 30 mM Tris, 10 mM glucose, 0.1 mM adenosine, pH 7.4). The cells were then incubated at 30°C for 15 min to allow cycles of endocytosis and exocytosis of media derived material to be completed. The incubation was terminated by the addition of 4 volumes of ice cold TES. The cells were pelleted by centrifugation (1000 x g, 10 min. at 4°C), and resuspended in ice-cold TES to a density of 5 x 10⁶ cells/mL. Cells were then incubated with 1:1000 rabbit anti-VSG 221 IgG antibody for 30 min on ice, washed 2X in ice-cold TES, and then incubated with 20 µg/mL of goat anti-rabbit IgG FITC-conjugated secondary antibody for 1 hour on ice. The cells were then washed 2X in TES, resuspended in TES at 5 x 10⁶ cells/mL, and transferred to a 96 well plate for analysis using the FLX800 fluorescence plate reader equipped with KC4 software. Kinetic analysis was performed by measuring fluorescent signal (excitation 490 emission 525) continuously every 2 min for 90 min at 37°C. Final fluorescent values were obtained by subtracting the “cell only” control from the treated cells.

Complement-mediated lysis assay

The complement-mediated lysis assay was carried out essentially as described by (Balber et al., 1979) as trypanolysis assay. VSG 221 expressing ACC-5 cells (induced and uninduced for ACC-RNAi) were grown for 9 days in low, normal, or high lipid media. Cells were harvested by centrifugation at 1000 x g and resuspended at 5 x 10⁶ cells/mL in BBSG. The cells were incubated with 1:1000 anti-VSG 221 rabbit IgG antibody in BBSG for 30 min on ice. The cells

were then washed 2X at 1000 x g for 5 min at 4°C. Cells with bound antibody were then incubated at 37°C for the time corresponding to the previously measured Δt determined for each lipid media (Table 2.1) to allow clearance of surface-bound antibody (17 min for normal media, 18 min for low lipid media, and 16 min for high lipid media). The cells were transferred to a 96 well plate and then incubated with 25% (final v/v) guinea pig complement reconstituted in BBSG containing 5 mM CaCl₂ and 5 mM MgCl₂. Cells were incubated in the presence of complement for 30 min. at 37°C. Cell survival was assessed by incubation of the cells with 0.05% (final v/v) Cell TiterBlue reagent for 60 min. Live cells can reduce the Cell Titer Blue dye reagent to a fluorescent product, which can be measured using the fluorescence plate reader (excitation 560, emission 590).

Figure 2.1. ACC-RNAi does not affect doubling time. **A.** BF ACC-RNAi cells grown in low, normal, and high lipid media were induced for ACC-RNAi for 3, 6, or 9 days. Total lysates (20 μ g protein) were probed for ACC by blotting with streptavidin-HRP (SA-HRP), which detects the ACC biotin prosthetic group (top panel), and with anti-tubulin antibody as a loading control (bottom panel). **B.** BF ACC-RNAi cells were seeded into low, normal, or high lipid media, induced for ACC-RNAi for 7 days, cell densities of induced (+RNAi) and uninduced control (No RNAi) cultures were recorded daily and used to calculate doubling time. Average doubling times \pm standard deviation are shown (n=3).

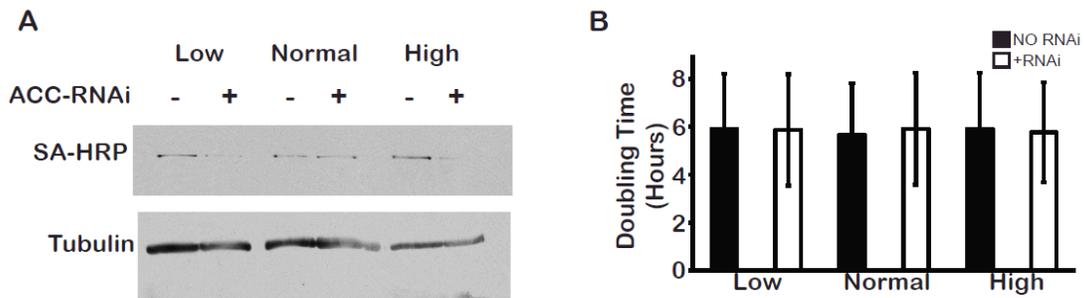
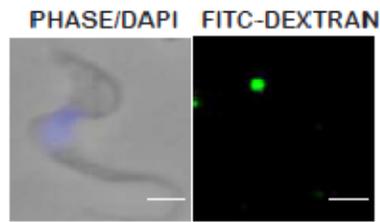
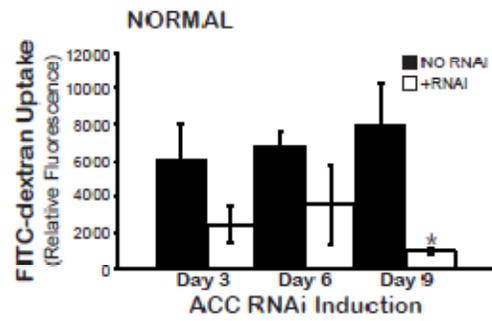


Figure 2.2. Fluid phase endocytosis is reduced in BF ACC-RNAi cells after 9 days for all growth conditions. **A.** BF ACC-RNAi cells were incubated with 150 µg/ml FITC-dextran for 10 min., fixed, permeabilized, and stained with 4',6'-diamidino-2-phenylindole (DAPI) to show nuclear and kinetoplast DNA, as described (Field et al., 2004). Representative microscopic image of Phase/DAPI overlay (left) and FITC-dextran fluorescence (right) (uninduced, normal media) is shown. Scale bar = 2 µm. **B-D.** BF ACC-RNAi cells were grown in normal, low, or high lipid media and induced for ACC-RNAi for 3, 6, or 9 days. Induced (+RNAi) and uninduced control (No RNAi) cells were incubated with 150 µg/ml FITC-dextran for 10 minutes at 37°C, lysed with 1% Triton-X-100, and fluorescence determined using a plate reader. Mean fluorescence +/- standard deviations is shown (n=3). Comparisons between uninduced and induced were performed using a 2-tailed Student's t-test. **B.** Normal media (*, p=0.04). **C.** Low lipid media (*, p=0.0003). **D.** High lipid media (*, p=0.05).

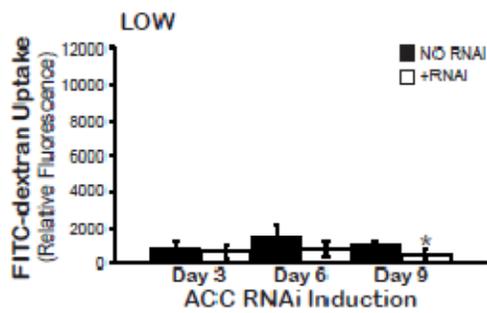
A



B



C



D

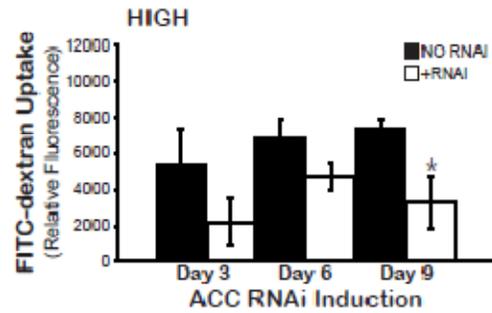


Figure 2.3. Receptor mediated endocytosis is reduced in BF ACC-RNAi cells after 9 days for all growth conditions. **A.** BF ACC-RNAi cells were incubated with 20 $\mu\text{g/ml}$ of transferrin-Alexa Fluor 488 for 10 minutes, fixed, permeabilized, and stained with DAPI as in Fig. 2A. Representative microscopic image of Phase/DAPI overlay (left) and Transferrin-Alexa 488 fluorescence (right) (uninduced, normal media) is shown. Scale bar = 2 μm . **B-D.** Uptake of 20 $\mu\text{g/ml}$ transferrin-Alexa Fluor 488 was performed essentially as described for the FITC-dextran uptake in Fig. 2B, except the lysis step was omitted because transferrin-Alexa 488 is not pH sensitive. Mean fluorescence \pm standard deviations is shown (n=3). Comparisons between uninduced and induced were performed using a 2-tailed Student's t-test. **B.** Normal media (*, p=0.05). **C.** Low lipid media (*, p = 0.02). **D.** High lipid media (*, p=0.007).

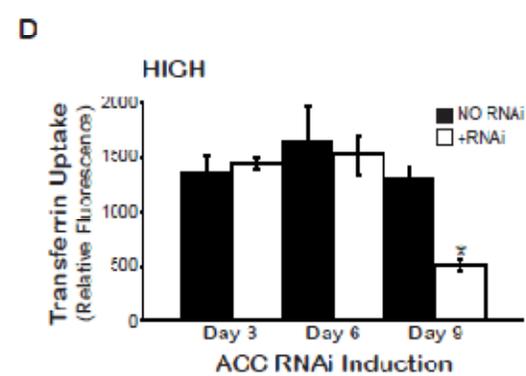
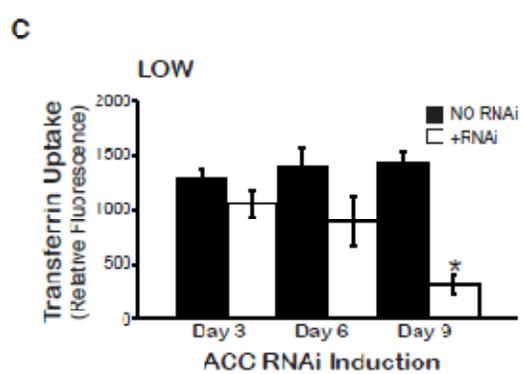
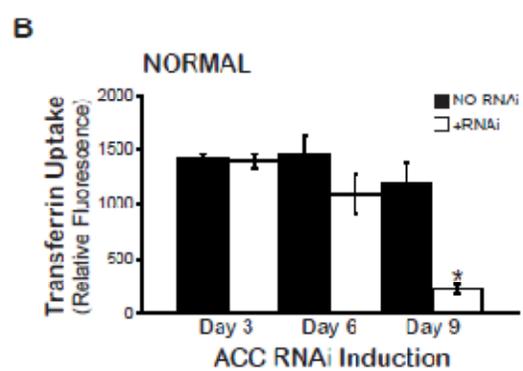
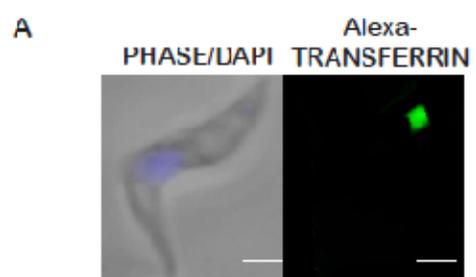


Figure 2.4. ACC-RNAi Slows Kinetics of Antibody Clearance from *T. brucei* surface. Clonal BF ACC-RNAi cells displaying the VSG 221 surface coat were isolated by flow cytometry and then induced for ACC-RNAi for 9 days in low, normal, or high lipid media. Induced (+RNAi) and uninduced control (No RNAi) cells were incubated with Anti-VSG 221 rabbit polyclonal antibody on ice for 30 min., followed by the addition of goat-anti-rabbit IgG FITC conjugated secondary antibody on ice for 1 hour. Cells were then transferred to a 37°C fluorescence plate reader and the FITC signal was recorded every 2 minutes continuously for 90 minutes at 37°C. Representative data for multiple replicates is shown (See Table 1). **A.** Cartoon of experimental timecourse indicating the endocytosis (decreasing fluorescence) and exocytosis (increasing fluorescence) phases of the timecourse. Δt is the total time from $t=0$ to the time it takes for exocytosed FITC-antibody to return to the initial fluorescence level before internalization. **B.** Low lipid media. **C.** Normal media. **D.** High lipid media.

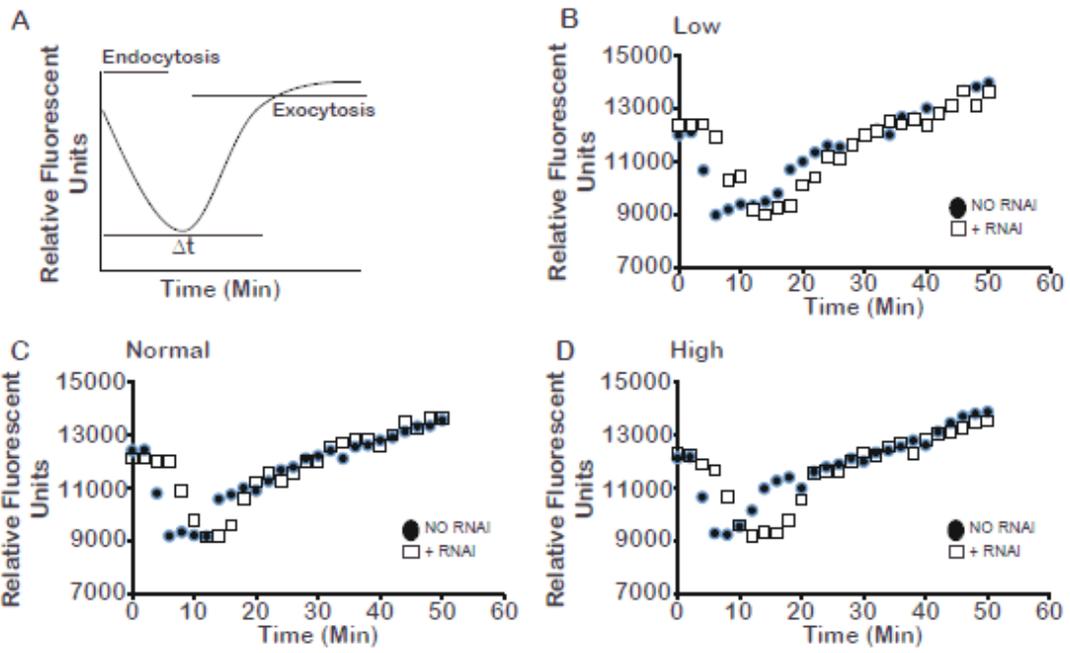


Figure 2.5. ACC-RNAi reduces the ability of *T. brucei* to avoid complement-mediated lysis. Clonal BF ACC-RNAi cells displaying the VSG 221 induced for ACC-RNAi for 9 days in low, normal, or high lipid media. Induced (+RNAi) and uninduced control (No RNAi) cells were incubated with Anti-VSG 221 rabbit polyclonal antibody on ice for 30 min., followed by an incubation at 37°C for empirically determined timepoints (Table 1): low, 18 mins, normal, 17 mins, and high, 18 mins. Cells were then incubated at 37°C in the presence of guinea pig complement. CellTiter-Blue Reagent was added to the cell solutions, which were transferred to a 37°C fluorescence plate reader and the signal was recorded after 60 minutes of incubation at 37°C. Mean fluorescence +/- standard deviations is shown (n=3). Comparisons between uninduced and induced were performed using a 2-tailed Student's t-test. Normal media (*, p=0.02). Low lipid media (*, p = 0.01). High lipid media (*, p=0.005).

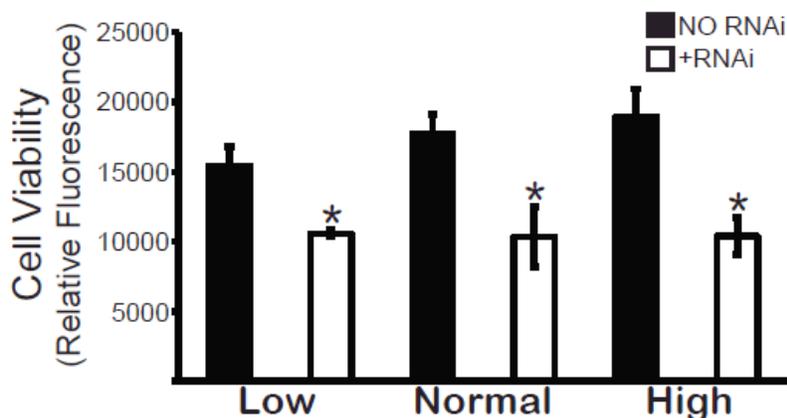


Table 2.1: Antibody Clearance is Delayed in BF ACC-RNAi Cells After 9 days for All Growth Conditions.

Growth Medium	ACC-RNAi	Δt^1 +/- standard deviation	N	p value²
Low Lipid	-	18 +/- 1.2	3	0.002
	+	24 +/- 0.89	3	
Normal Lipid	-	17 +/- 1.03	3	0.002
	+	23 +/- 1.11	3	
High Lipid	-	16 +/- 0.98	3	0.003
	+	22 +/- 1.21	3	

¹ Δt is the calculated average of time of antibody clearance via endocytosis and exocytosis of the surface bound FITC conjugated secondary antibody.

²Significance of comparison of Δt of uninduced and induced BF ACC-RNAi for each growth media was determined using a 2-tailed Student's t-test.

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LIPID DROPLET MAINTENANCE IN *T. brucei*:
THE EFFECT OF FATTY ACID SYNTHESIS, UPTAKE, AND
ENVIRONMENTAL LIPIDS

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ABSTRACT

Trypanosoma brucei, the causative agent of human African trypanosomiasis (HAT), is a protozoan parasite that lives extracellularly within the mammalian host, where it must adapt to a variety of host micro-environments that differ in nutrient content. *T. brucei* is able to supply itself with fatty acids in two manners: uptake from the environment or synthesis. The parasites can also store fatty acids in the form of neutral lipids storage within organelles known as lipid droplets. To begin characterizing lipid droplets in *T. brucei*, we examined the effect of environmental lipid availability on the lipid droplets. We found a $62 \pm 2\%$ decrease in lipid droplet number when parasites were grown in a low lipid media, but observed no change in lipid droplets in high lipid media. Next, we inhibited fatty acid synthesis through RNAi of the Acetyl-CoA Carboxylase, which catalyzes the first committed step in fatty acid synthesis. ACC RNAi for 3 days resulted in a decrease in lipid droplet number by $29 \pm 5\%$, $34 \pm 8\%$, and $20 \pm 5\%$ in normal, low, and high lipid media respectively. ACC RNAi for 9 days increased

this effect, reducing lipid droplet number by $57 \pm 15\%$, $37 \pm 15\%$, and $27 \pm 4\%$ in normal, low, and high lipid media, respectively. Finally, addition of exogenous palmitic acid (C16:0) to BF parasites induced and uninduced for ACC-RNAi grown in low lipid medium for 9 days to determine the effect on lipid droplet number. As we observed before, ACC-RNAi decreased lipid droplet number in all three conditions. However, exogenous addition of either a fluorescent BODIPY-conjugated or unconjugated fatty acid, palmitate (C16:0), resulted in a greater than 2-fold increase in lipid droplet number. This leads us to believe that fatty acid synthesis, uptake, and the environmental lipid availability all act in concert to affect lipid droplet maintenance/biogenesis in *T. brucei*.

INTRODUCTION

Trypanosoma brucei is an extracellular protozoan parasite vectored by the tse tse fly that infects both humans and livestock in sub-Saharan Africa. It causes human African trypanosomiasis (HAT) in humans and a wasting disease, nagana, in cattle. Within its hosts, *T. brucei* must adapt to many distinct environments, differing in available nutrients. For example, there are ~300X fewer available lipids in the cerebrospinal fluid compared to the bloodstream (Lentner, 1981). *T. brucei* has a large fatty acid requirement because they are constituents of glycosylphosphatidylinositol (GPI) anchors, which anchor key glycoproteins to the surface. The mammalian bloodstream form (BF) parasite is covered in variant surface glycoproteins (VSGs), each requiring two myristates

(Paul *et al.*, 2001), while the insect procyclic form (PF) parasite is covered in procyclins, each containing two fatty acids (Acosta-Serrano *et al.*, 2001).

T. brucei is able to supply itself with fatty acids in two distinct manners: *de novo* synthesis of fatty acids or uptake from the environment. Although, uptake is more energetically favorable, *T. brucei* must rely on its fatty acid synthesis (FAS) pathways when environmental fatty acids are limiting (Lee *et al.*, 2006; Vigueira and Paul, 2011). Two FAS pathways have been described in *T. brucei*. The elongase (ELO) pathway is found in the ER and accounts for the majority (~90%) of *de novo* synthesis (Lee *et al.*, 2006) and the mitochondrial pathway (~10%) (Stephens *et al.*, 2007; Guler *et al.*, 2008). The first committed step of fatty acid synthesis is the conversion of malonyl-CoA to acetyl-CoA via the enzyme acetyl-CoA carboxylase (Tehlivets *et al.*, 2007; Saggerson, 2008). Previously RNA interference (RNAi) of *T. brucei* ACC, demonstrated that the enzyme is necessary for full virulence in murine models (Vigueira and Paul, 2011). We hypothesize that this necessity is based on the reliance of *T. brucei* on fatty acids in host adaptation, specifically in lipid droplet maintenance/biogenesis.

Lipid droplets are highly dynamic lipid storage organelles found primarily in eukaryotes but also in some prokaryotes (Law and Wells, 1989; Packter and Olukoshi, 1995; Hsieh and Huang, 2005; Ashraft, 2007). Lipid droplets are usually composed of neutral lipids. The most common storage lipids are triacylglycerols and cholesterol esters, but fatty acids and diacylglycerols also have been identified in the core of these droplets (Martin and Parton, 2006;

Czabany *et al.*, 2008; Goodman, 2008; Reue, 2011). Surrounding the neutral lipid core is a phospholipid monolayer membrane similar to that of the ER, with the acyl chains facing inward (Dvorak *et al.*, 1994; Wan *et al.*, 2007; Goodman, 2009). Lipid droplets are involved in energy storage, lipid metabolism, lipid trafficking, and protection against toxic effects of non-esterified lipids and sterols (Murphy, 2001; Ozeki *et al.*, 2005; Farese *et al.*, 2009), but the extent of the involvement in all of these activities is highly specific to species, tissue, and/or cell type.

Lipid droplet synthesis and characterization in *T. brucei* is an unexplored area of research. It is unknown how lipid droplets are created and trafficked within the cell and what proteins may be involved in the process or colocalized with lipid droplets. One kinase, lipid droplet kinase (LDK), was recently found to be associated with lipid droplets in *T. brucei*. LDK is on or embedded in the phospholipid monolayer of the lipid droplets and is involved in both the synthesis and the maintenance of lipid droplets (Flaspohler *et al.*, 2010).

We hypothesize that FAS, uptake, and environmental lipid concentration effect *T. brucei* lipid droplets. Since lipid droplets serve as a storage organelle and may be involved in trafficking, we hypothesize that newly synthesized fatty acids can be stored within the lipid droplets and that the lipid droplets may serve as a reservoir, from which the parasite can utilize fatty acids for lipid biogenesis.

First, we examined the environmental effect of lipid concentration on the lipid droplets of *T. brucei*. In order to manipulate the lipid concentration of the

media, we altered the serum levels as previously described (Vigueira and Paul, 2011). The low lipid medium contained ~4% lipid equivalents, normal lipid medium ~ 12%, and high lipid medium ~22%. We found a reduction in lipid droplet number after prolonged growth in the low lipid medium. Then, we utilized the BF ACC-RNAi cells grown in the three different lipid concentrations of media to further decipher the effects of environmental lipids and the loss of fatty acid synthesis on lipid droplet number. We found that under all conditions, there was a significant reduction in lipid droplet number when ACC-RNAi was induced. Lastly, we determined the effect of exogenous fatty acids on lipid droplet number. When parasites were grown in low lipid medium and then supplemented with palmitic acid (C16:0), there was an increase in lipid droplet number, suggesting that fatty acids from the environment are trafficked to the lipid droplets. In summary, the environmental status of lipids, FAS, and uptake are all important in lipid droplet maintenance.

RESULTS

The effect of growth medium on lipid droplet number

It has been previously determined that exogenous lipid content in media affects lipid droplet number. Growth in high lipid containing media, usually results in an increase in lipid droplet number or size (Tachi-Sato *et al.*, 2002). While growth in low lipid media for a short period of time (<24h), also results in an increase in lipid droplet number or size (Flaspohler *et al.*, 2010). We assessed the effect of growth medium on lipid droplet number in *T. brucei*. After 3 days of

growth in low, normal, and high lipid media, the number of lipid droplets were not different between parasites grown in normal and high lipid media; however, there was a $62\pm 2\%$ decrease in the number of lipid droplets of parasites grown in low lipid media versus normal lipid media (Fig. 2.1). We were also able to recapitulate the results where growth in low lipid media for ~24h increases lipid droplet numbers (data not shown).

The effect of ACC-RNAi on lipid droplet number

The induction of ACC-RNAi knocks down protein expression in BFs (Fig. 2.1A), but when doubling time is analyzed in BF ACC-RNAi induced and uninduced parasites, there is no difference in doubling rate (Fig. 2.1B). *T. brucei* is able to maintain an internal lipid store in the form of lipid droplets (Flaspohler *et al.*, 2010). Since we subjected the parasites to growth in different lipid containing medium and reduced its capability to undergo fatty acid synthesis, we assayed how the lipid droplet number is affected under such conditions. In normal lipid medium, there is a $29\pm 5\%$ reduction in lipid droplet number after 3 days of growth and induction of ACC-RNAi, $48\pm 11\%$ reduction after 6 days growth and induction, and $57\pm 15\%$ reduction after 9 days of growth and induction (Fig. 3.2A). Similarly, there is a $34\pm 8\%$ reduction in lipid droplet number after 3 days of growth and induction of ACC-RNAi in low lipid medium, $25\pm 18\%$ reduction after 6 days of growth and induction, and $40\pm 15\%$ reduction after 9 days of growth and induction (Fig. 3.2C). There is also a $20\pm 5\%$ reduction in lipid droplet number after 3 days of growth and ACC-RNAi induction in high lipid medium, $16\pm 2\%$

reduction after 6 days of growth and induction, and $27\pm 4\%$ after 9 days of growth and induction (Fig. 3.2E).

The effect of the addition of exogenous Fatty acids on lipid droplet number

We determined that long term growth in low lipid medium decreases lipid droplet number (Fig. 3.1). We also determined that ACC-RNAi decreases lipid droplet number (Fig. 3.2). Since the environment and disruption of the major *de novo* synthesis pathway decreases lipid droplet formation/maintenance, we examined the effect of adding back exogenous fatty acids on lipid droplet number on No RNAi and +RNAi parasites that were grown in low lipid medium for 6 days. Further, we wanted to determine where the exogenous fatty acids localize within the cell, specifically if they went to the lipid droplets. It was previously determined that BFs readily take up a Bodipy conjugated palmitic acid (Bodipy-C16) at a concentration of $20\ \mu\text{M}$ at 37°C for 20 min (Howell and Paul, Unpublished). To be sure that the fatty acid component and not the Bodipy dye were being trafficked to lipid droplets, we incubated parasites with $20\ \mu\text{M}$ unlabeled palmitic acid (C16:0) dissolved in methanol. There were still significant reductions in each condition for ACC-RNAi induced BFs, but there was a significant increase in the add back samples, i.e. the Bodipy-C16 and the unlabeled-C16. There was a $227\pm 100\%$ increase in the Bodipy-C16 addition from uninduced low lipid medium and a $229\pm 112\%$ increase in the unlabeled-C16 addition (Fig. 3.3). Additionally, there was also an increase in the induced parasites, although they still had a lipid droplet number defect. There was a $262\pm 100\%$ increase in the Bodipy-C16

addition from the induced low lipid medium and a $260\pm 35\%$ increase in the unlabeled-C16 addition (Fig. 3.3). There were no differences between the Bodipy-FL only control (Bodipy conjugated to C2), methanol control, DMSO control, and the standard BFs grown in low lipid medium for 6 days (Fig. 3.3).

DISCUSSION

Here we demonstrate that the environmental status of lipids, FAS, and uptake influence lipid droplet maintenance and biogenesis. Incubation in low lipid medium for an extended period of time (3 days or more) resulted in depleted lipid droplet numbers. ACC-RNAi decreased lipid droplet numbers in all lipid media. Further, the addition of exogenous C16 to low lipid medium increased lipid droplet number and demonstrated that fatty acids are trafficked to the lipid droplet.

The environmental lipid content both positively and negatively affects lipid droplet number. Initially, short-term exposure (up to 24 hours) to low lipid environment increases the lipid droplet numbers, this is due in part to the upregulation of FAS (Lee *et al.*, 2006; Flaspohler *et al.*, 2010). However, prolonged growth in low lipid environments depletes lipid droplets, suggesting a utilization of internal lipid stores to maintain lipid biogenesis (Goodman, 2008). In high lipid environments, there was no increase in lipid droplet numbers from normal lipid medium. This could be due to a threshold value of lipid droplet number that the cell holds constant, meaning that the cell does not need or make any more lipid droplets.

Since environmental lipid status affects both lipid droplet number and FAS, we determined the effect of ACC-RNAi on lipid droplet number. ACC-RNAi effectively reduced the number of lipid droplets in all lipid media. Therefore, FAS and ACC is important in the maintenance and biogenesis of lipid droplets, regardless of environmental lipid status. Previously, Flaspohler *et al.*, demonstrated that LDK is important in lipid droplet biogenesis and maintenance by demonstrating that there is a decrease in lipid droplet number even when environmental conditions would induce an increase (Flaspohler *et al.*, 2010). We also saw a decrease in lipid droplet number in known elevating conditions. Even with a short exposure to low lipid medium, there was still a decrease in ACC-RNAi parasites compared to uninduced (Data not shown).

Since ACC is important in lipid droplet maintenance, we hypothesize that lipid droplets may serve as a reservoir of fatty acids and lipids to feed into lipid biogenesis for production of membrane lipids and lipid signaling molecules. To test this hypothesis, we wanted to demonstrate that exogenous fatty acids, when the cell has been grown in low lipid medium for an extended period of time, are trafficked to lipid droplets. We did determine that exogenous fatty acids are brought into the cell and are eventually trafficked to lipid droplets, increasing their numbers. Since exogenous fatty acids are stored in lipid droplets, we hypothesize that fatty acids are taken up or synthesized and shuttled to the lipid droplet, where they can be utilized in lipid biogenesis. Other neutral lipid products from either uptake or synthesis may also be stored or utilized from lipid droplets.

Therefore, we hypothesize that lipid droplets in *T. brucei* are truly dynamic organelles that associate with the trafficking pathways, much like lipid droplets in yeast, adipocytes, hepatocytes, and muscle cells (Novikoff *et al.*, 1980; Blanchette-Mackie *et al.*, 1995; Murphy and Vance, 1999; Habeler *et al.*, 2002; Cohen *et al.*, 2004; Binns *et al.*, 2006; Bartz *et al.*, 2007; Guo *et al.*, 2008; Goodman, 2008; Goodman, 2009; Reue, 2011).

Our preliminary data supports our hypotheses; however, we will need to perform some colocalization assays to further prove that fatty acids are trafficked to lipid droplets. We also need to complete live cell imaging to solidify the trafficking pattern into and within the cell of our fatty acids. And lastly, we will need to determine the changes if any in bulk lipid synthesis to connect FAS and lipid droplet depletion to bulk lipid synthesis.

Lipid droplets are becoming more important in biomedical research. Some cancer cells present larger, more numerous lipid droplets. Additionally, diabetes research and muscular disorders research are also highly interested in lipid droplet biology (Dvorak *et al.*, 1983; Murphy, 2001; Melo *et al.*, 2011; Haemmerle *et al.*, 2011). Lipid droplets become increasingly interesting in *T. brucei* because their primary usage is obsolete within the parasite. Although *T. brucei* possesses the machinery to undergo β -oxidation, no one has described the process in BFs. The only energy metabolism that occurs in BFs is glycolysis. Therefore, *T. brucei* offers a unique study organism for lipid droplet biology. Since it does not utilize

lipid droplets as an energy storage, it could be easier to determine their roles in the trafficking pathways in the cell and associations to other organelles.

Future Directions

In the future, I suggest we begin to delve into the relationship between FAS and lipid droplet maintenance in and biogenesis by doing some studies with the LDK mutant lines from Dr. Parson's group. We need to investigate ACC levels and phosphorylation states in the LDK-RNAi lines to determine if the two pathways work in concert or antagonistically.

Currently, we are investigating other potential proteins that could be involved in lipid droplet maintenance by screening the RNAi library available in PFs. If we can find other proteins involved in lipid droplet maintenance, we should also look at ACC levels and phosphorylation to determine linkage between the pathways.

More interestingly, we made a preliminary connection between fatty acid uptake and lipid droplet maintenance. We demonstrated that exogenous fluorescently tagged fatty acids do become incorporated into lipid droplets. However, we should conduct live cell imaging with our Bodipy tagged C16 to further elucidate the downstream uptake pathway, i.e. can we track fluorescent C16 through the endocytic pathway to the ER and then the lipid droplet and beyond?

Lastly, we should determine the effect of lipid droplet depletion and ACC-RNAi on bulk lipid synthesis in the cell. I began a preliminary experiment with this

utilizing a “cold” TLC method. We should move on to “hot” TLC, utilizing ^3H fatty acids to track lipid biogenesis and determine the differences if any in our ACC-RNAi lines and environmental effects on bulk lipid synthesis.

ACKNOWLEDGMENTS

We thank Dr. Marilyn Parsons for helpful discussions and XYZ for critical reading of the manuscript. This work was supported by funds from the NIH (R15AI081207-01A1).

MATERIALS AND METHODS

Reagents

All chemicals and reagents used in these studies were purchased from Thermo Fisher Scientific and Sigma except Iscove’s Modified Dulbecco’s Medium (IMDM), Bodipy conjugated fatty acids (C16:0 and C2:0), and 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen), Serum Plus (JRH Biosciences), delipidated fetal bovine serum (FBS) (Cocalico Biologicals), and poly-L-lysine solution (Electron Microscopy Sciences).

Trypanosome Strains and Cell Lines

Previously, BF and PF ACC-RNAi cell lines were generated as described from BF 90-13 cell lines or PF 29-13 cell lines (Wang *et al.*, 2000; Morris *et al.*, 2001; Vigueira and Paul, 2011). The BF 90-13 ACC-5 cell lines were grown in HMI-9 medium (Hirumi and Hirumi, 1989) containing 10% heat-inactivated FBS/10% Serum Plus and supplemented with 2.5 $\mu\text{g/ml}$ G418, 5 $\mu\text{g/ml}$

hygromycin, and 2.5 µg/ml phleomycin. PF ACC-RNAi cell lines were grown in SDM-79 medium (Brun and Shonenberger, 1979) containing 10% heat-inactivated FBS and supplemented with 15 µg/ml G418, 50 µg/ml hygromycin, and 2.5 µg/ml phleomycin.

Streptavidin Blotting

Hypotonic lysates were prepared as previously described (Morita et al., 2000). Streptavidin blotting can detect the biotin prosthetic group on ACC and was performed essentially as described (Vigueira et al., 2011). Briefly, the lysates were fractionated on 10% SDS-PAGE gels, transferred to nitrocellulose, and blocked in Wash Buffer (1% dry milk, 1X TBS, 0.05% Tween-20). The blot was then cut; the top was half probed for ACC with SA-HRP and the bottom for tubulin. The blot was probed for ACC with streptavidin-horseradish peroxidase conjugate (1:400 in 0.2% dry milk, 1X Tris-buffered saline (TBS), 0.05% Tween-20). The lower portion was probed for tubulin with a mouse anti-tubulin antibody (clone B-5-1-2) diluted 1:50,000 in Wash Buffer followed by HRP-conjugated goat anti-mouse IgG antibody (Invitrogen) diluted 1:10,000 in Wash Buffer. Semi-quantitative analysis of the blots was performed using densitometry (NIH Image J software) of exposed films (unsaturated signal within the linear range of the film).

Growth Curves

Bloodstream form ACC-RNAi cells were diluted into normal, low, or high lipid media at 10^4 cells/mL, induced by the addition of tetracycline (1 µg/mL final)

(Wang *et al.*, 2000), and cell density monitored using a hemocytometer. For comparison, the slopes of the growth curves (linear correlation coefficients) were derived from linear regressions performed using Microsoft Excel. Doubling times were calculated from the slopes.

Preparation of Low Lipid and High Lipid Medias

As previously reported, the only source of lipids in media is from the serum additives (Vigueira and Paul, 2011). Serum Plus and delipidated FBS contain ~20% serum lipids. Low-lipid HMI-9 media was prepared with 10% Serum Plus and 10% delipidated FBS. The resulting media contain serum lipids equivalent to 4% (Vigueira and Paul, 2011). High-lipid HMI-9 media was prepared with 10% Serum Plus and 20% FBS, resulting in a media containing serum lipids equivalent to 22% (Vigueira and Paul, Unpublished?). Low lipid medium was prepared with 10% delipidated FBS, and contained serum lipids equivalent to 2% FBS, compared to 10% for the normal SDM-79 (Vigueira and Paul, 2011). High lipid medium was prepared with 20% FBS, and contained serum lipids equivalent to 20% FBS.

Lipid Droplet Microscopy

Microscopy was performed essentially as described in (Field *et al.*, 2004). Briefly, BF ACC-RNAi uninduced and induced cells were grown in normal, low, and high lipid media for 3, 6, or 9 days. They were harvested by centrifugation (800 x g, 10 min) and washed 1 time in ice-cold Voorheis's modified PBS (vPBS)

(Nolan et al., 2000). The cells were fixed on ice in 1.5% paraformaldehyde (w/v) in vPBS for 10 minutes. Fixed parasites were adhered to poly-L-lysine treated glass slides and permeabilized for 10 minutes in 0.1% Triton X-100 (v/v) in vPBS. The cells were then incubated with 0.05% Nile Red diluted in vPBS (Greenspan *et al.*, 1985; Robibaro *et al.*, 2002). The fluorescence of Nile Red is more specific to lipid droplets in the FITC channel (528 nm); therefore, data collected was done so in the green channel (Listenberger, *et al.*, 2007; Flaspohler *et al.*, 2010). For Bodipy-C16 uptake experiments, microscopy was performed essentially the same; however, the cells were pre-incubated before fixation with vPBS only control, 20 μ M methanol only control, 20 μ M DMSO only control, 20 μ M Bodipy-C16 in vPBS, 20 μ M Bodipy-FL in vPBS, or 20 μ M unlabeled C16:0 dissolved in ethanol. The nucleus and kinetoplast were stained with 4'-6-diamidino-2-phenylindole (DAPI) (1 mg/mL in vPBS) (Invitrogen) (Field et al., 2004). Lipid droplets were counted per nuclei for 100 nuclei. These assays were repeated 3 times. Student t-tests were performed utilizing Microsoft Excel. Images were collected using a Nikon TE2000 widefield epifluorescence microscope and image acquisition was performed using Nikon NIS Elements software package. Image analysis was further performed using NIH Image J and Adobe Photoshop.

Figure 3.1. Exogenous Lipid Content Affects the Number of Internal Lipid Droplets. Fixed and permeabilized BFs were stained with Nile Red to detect lipid droplets and with 4',6'-diamidino-2-phenylindole (DAPI) to stain nuclei and kinetoplast DNA (kDNA) and imaged as described (Robibaro et al., 2002 and Field et al., 2004). The number of lipid droplets per nucleus were counted and the average value \pm standard deviation is shown (100 nuclei, n=3). *, p<0.0001, 2-tailed Student's t-test, low vs. normal.

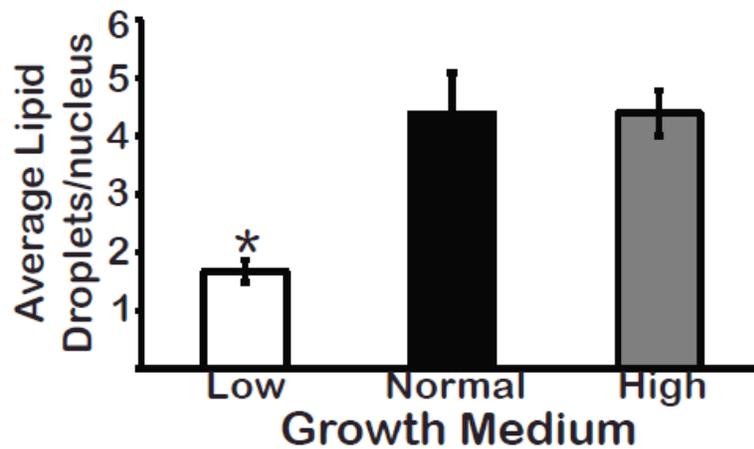


Figure 3.2. ACC-RNAi Decreases the Number of Lipid Droplets in *T. brucei* in All Growth Conditions. BF ACC-RNAi cells were grown in normal, low, or high lipid media and induced for ACC-RNAi for 3, 6, or 9 days. Induced (+ RNAi) and uninduced controls (No RNAi) were stained with Nile Red (lipid droplets) and DAPI (nuclei and kDNA) as described in Fig. 2. **A, C, and E.** Average lipid droplet per nucleus \pm standard deviation (100 nuclei, n=3). Statistical comparisons between uninduced and induced were performed using 2-tailed Student's t-test. **A.** ACC RNAi induction in normal media (**, $p < 0.0001$). **C.** ACC RNAi induction in low lipid media (*, $p = 0.002$; **, $p < 0.0001$). **E.** ACC RNAi induction in high lipid media (**, $p < 0.0001$). **B, D, and F.** Representative microscopy for each a 9 day ACC RNAi induction in each growth medium. Phase/DAPI overlay (left) and Nile red fluorescence (right) are shown. Scale bar = 2 μm . **B.** Normal media. **D.** Low lipid media. **F.** High lipid media.

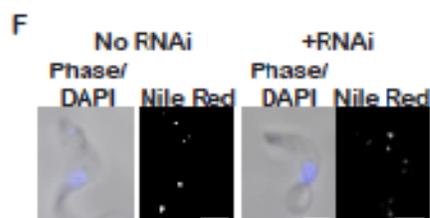
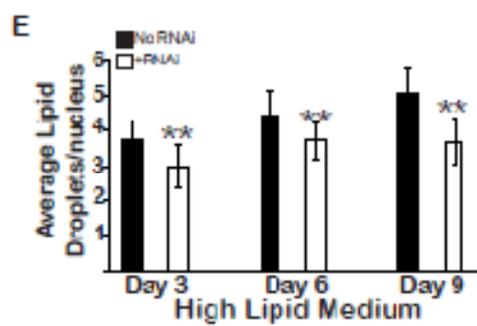
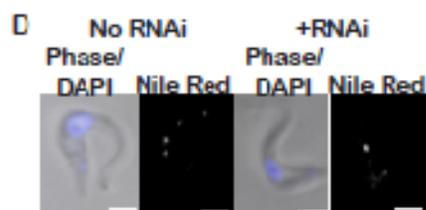
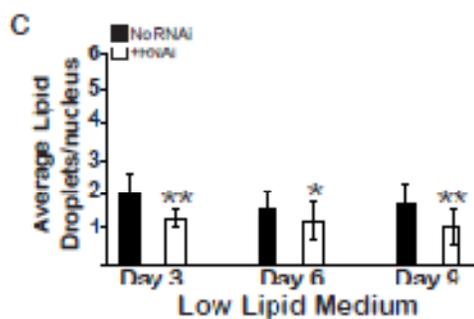
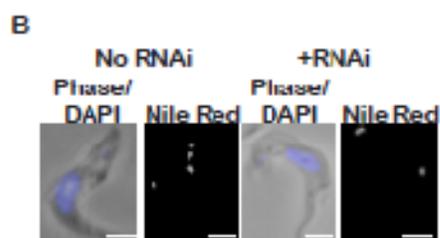
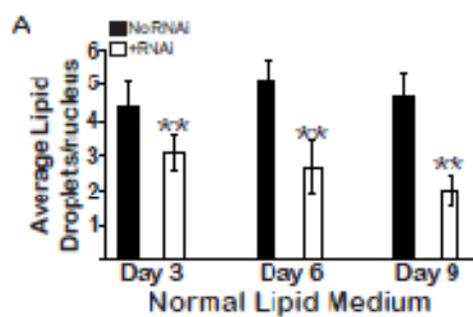
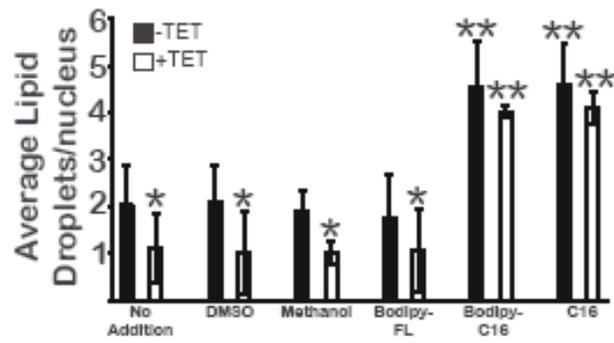
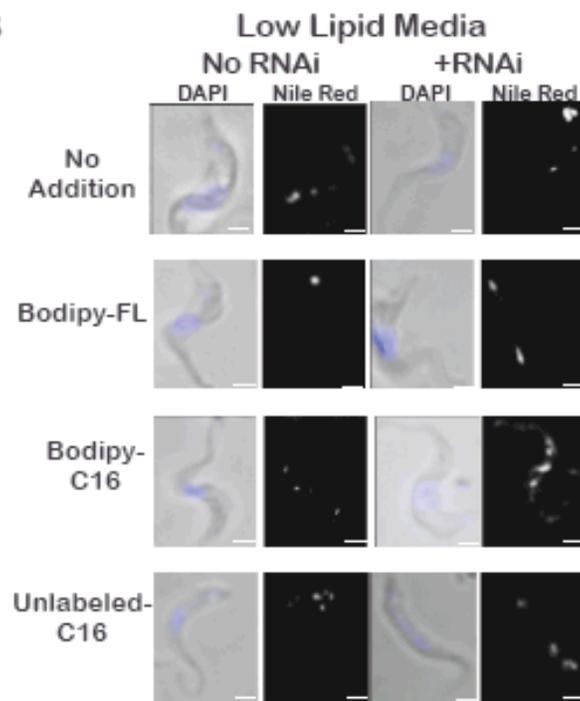


Figure 3.3. Addition of Exogenous Palmitic Acid (C16:0) Increases Lipid Droplet Number. BF ACC-RNAi cells grown in low lipid media were induced for ACC-RNAi for 9 days. Induced (+ RNAi) and uninduced control (No RNAi) cells were incubated with no fatty acid, 20 μ M unlabeled C16 in ethanol, 20 μ M Bodipy-FL, or 20 μ M Bodipy conjugated C16 for 20 minutes at 37°C. Cells were then imaged and lipid droplet number per nucleus determined as described in Fig. 2 (100 nuclei, n=3). **A.** Average lipid droplets per nuclei are shown \pm standard deviations. Comparisons between uninduced and induced were performed using a 2-tailed Student's t-test. **B.** Representative microscopy of each condition. Phase/DAPI overlay and Nile red fluorescence are shown. Scale bar 2 μ m.

A**B**

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CHAPTER 4

CONCLUSIONS

Lipid metabolism in *Trypanosoma brucei* is a unique and essential process, which makes it an excellent metabolic pathway to study. The parasite's major *de novo* synthesis pathway found in the ER, utilizing a series of microsomal elongases, is novel, and many of the characterized genes represent potential drug targets. Since there is a plethora of gene targets to explore in this pathway, it is surprising that lipid metabolism remains an understudied subject in *T. brucei*.

The research that I have presented in this thesis has added to our knowledge of lipid metabolism in *T. brucei*. I have elucidated a novel role for acetyl-CoA carboxylase (ACC) in virulence, specifically the upregulation of endocytosis to clear surface immune antigens. However, we did not attempt to describe the role of ACC in antigenic variation. In the future, variant surface glycoprotein (VSG) presentation on the cell surface and switching should be determined in ACC mutants to further define the role of ACC in virulence. It may also be interesting to harvest trypanosomes from mice infected with the ACC mutant lines and compare the populations' VSG heterogeneity to that of mice infected with control parasites to determine if there is a difference in switch rates *in vivo*. If we can connect reduction of ACC activity to a reduction in multiple immune evasion tactics, targeting this enzyme could become a fruitful endeavor as a potential drug target.

Not only did we investigate the role of ACC in virulence, we also started to characterize lipid droplet biology in *T. brucei*. Only one study on lipid droplets in *T. brucei* is published. My studies linked fatty acid synthesis, environmental lipid availability, and fatty acid uptake to lipid droplet dynamics. Because lipid droplets could serve as an important source of lipids for the parasite, especially in low lipid environments (i.e. cerebrospinal fluid), elucidating the role of these lipid stores *in vivo* could help us to understand the parasite's ability to adapt to its host.

There are also a number of other reasons to study lipid droplets. They are becoming increasingly important in other branches of biomedical research, i.e. cancer, diabetes, energy acquisition in muscle cells during rest and exercise, and neurological messaging/ myelin sheath repair research. Since all eukaryotic cells and some prokaryotes possess lipid droplets, they are a dynamic and diverse organelle to study. To better understand lipid droplets in *T. brucei*, the lab has initiated an undergraduate research project on lipid droplets under my supervision. We are screening the RNAi library for other genes linked to lipid droplet maintenance/biogenesis in *T. brucei*. This screen will allow us to begin unraveling the molecular and regulatory mechanisms mediating lipid droplet biology within the African trypanosome.

As for other future endeavors, another important line of investigation will be to examine fatty acid uptake in *T. brucei*. Because *T. brucei* is able to supply itself with fatty acids through both uptake and synthesis, I believe it is important

to examine these two acquisition mechanisms in tandem. If fatty acid synthesis or uptake provides a drug target, certainly there should be a two-pronged attack targeting both parts of the supply chain.

I hope that this thesis provides useful information for future researchers interested in *T. brucei*. I believe that the continuation in defining lipid metabolism in *T. brucei* could help to bring forth a cure for a devastating disease that has too longed plagued sub-Saharan Africa.