2013-01-01

Giardia lamblia: Regulation of Cyst Production by Glycosphingolipids

Tavis Lyle Mendez
University of Texas at El Paso, tlmendez@miners.utep.edu

Giardia lamblia: Regulation of Cyst Production by Glycosphingolipids

Tavis Lyle Mendez

Department of Biological Sciences

APPROVED:

Siddhartha Das, Ph.D., Chair

Igor C. Almeida, D.Sc.

Rosa A. Maldonado, Ph.D.

Renato Aguilera, Ph.D.

German Rosas-Acosta, Ph.D.

Ricardo Bernal, Ph.D.

Benjamin C. Flores, Ph.D.
Dean of the Graduate School

Dedication

I dedicate this thesis to my brother Dennis Dwight Mendez

Giardia lamblia: Regulation of Cyst Production by Glycosphingolipids

by

Tavis Lyle Mendez, B.S.

DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

August 2013

Acknowledgements

I would like to acknowledge everyone in the world. Special thanks to my wife Leobarda Robles, my mother Masako Dobashi, my grandparents Nobuko and Hatsumoto Dobashi, the Resler family and my step-mother Sandra Lee Mendez. Their undying support has had a profound effect on me. The Das lab members Trevor Duarte, Atasi De Chatterjee, Yuni Hernandez, Debashi Roy, Mayte Yichoy, Danny Gomez, Nathaniel Ng, Isela Arrieta, Kristina Aguilera, Joaquin De Leon, Monica Delgado, Raymond Thomas Jones and other past and present members have been instrumental for my time here at UTEP. Felipe Gazos Lopes, Armando Varela and Gladys Almodovar have also been a great help when I needed them most. My committee members Siddhartha Das, Igor Almeida, Rosa Maldonado, Renato Aguilera, German Rosas-Acosta and Ricardo Bernal have my greatest thanks for their work in making my project one that I have enjoyed pursuing.

Honorable mention goes to Siddhartha Das and Renato Aguilera for giving me a paycheck through NIH-R01 and RISE, respectively.

Abstract

The diplomonad protist, *Giardia lamblia*, colonizes and replicates in the small intestine of mammals. In humans, *Giardia* infection (known as giardiasis) can be symptomatic or asymptomatic with the former being associated with fatty stool, abdominal pain, malnutrition, and weight loss. Both cell-mediated and humoral immune responses against *Giardia* infection are possible, and adaptive responses have been reported to be important for controlling the infection. Non-immune components such as secretory immunoglobulin, intestinal lipids, and bile acids also play significant roles in determining the severity of the infection by *Giardia*.

Giardia exists in two morphologic forms—trophozoites and cysts—and maintains a simple life cycle. Exposure of cysts to gastric acid during passage through the human stomach triggers excystation (morphological changes from cyst to trophozoites), while factors in the small intestine, where trophozoites colonize, induce encystation or cyst formation. The disease is transmitted by water-resistant cysts, which are excreted in the feces of humans and pass on to a new host via contaminated food and water. The production of viable giardial cysts is critical for successful transmission and spreading of the disease.

Our laboratory is interested in investigating the lipid metabolism in *Giardia* because this parasite is unable to synthesize the majority of its own lipids de novo and depends on exogenous lipids for its growth and encystation. In this regard, I am interested in identifying the role of sphingolipids (SLs) during cyst formation. We have shown earlier that, unlike higher eukaryotes *Giardia* expresses fewer SL genes, which are differentially regulated during encystation. This suggests that there could be a link between SL synthesis and giardial encystation. Furthermore, D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), an inhibitor of glucosylceramide (GlcCer) synthesis, blocks replication of trophozoites, induces karyokinesis, and inhibits cyst production by *Giardia*, which indicates that GlcCer plays an essential role in the life cycle of this waterborne pathogen.

The goal of my dissertation is to provide understanding of the overall process of cyst production and how the enzyme of GlcCer synthesis (i.e., GlcT1) modulates this process. I have used various molecular and biochemical methodologies to address this question with the help of two separate but

interrelated Specific Aims. In Specific Aim 1, I asked if giardial glucosylceramide transferase enzyme (gGlcT1) regulates encystation and cyst production by *Giardia*, and the goal of Specific Aim 2 was to investigate whether glucosylceramide transferase regulates ESV (encystation-specific vesicle) biogenesis and cyst viability by maintaining the cellular lipid homeostasis.

Briefly, I found that overexpression of gGlcT1 enzyme generated aggregated/enlarged ESVs and congregated cysts with reduced viability. The knocking down of gGlcT1 activity blocked ESV biogenesis completely and produced mostly non-viable cysts. However, when gGlcT1-overexpressed *Giardia* was knocked down with anti-gGlcT1 morpholino oligonucleotide (i.e., GlcT1 rescued parasites) the enzyme activity, ESV biogenesis, and cyst viability returned to normal, suggesting that the regulated expression of gGlcT1 is important for encystation and viable cyst production. Overexpression of gGlcT1 increased the influx of membrane lipids and fatty acids without altering the fluidity of plasma membranes, which indicates that the expression of gGlcT1 is linked to lipid internalization and maintenance of the overall lipid homeostasis in this unicellular organism. Based on these results, I propose that gGlcT1 is a key player in ESV biogenesis and *Giardia* cyst viability and that it could therefore be exploited for developing new anti-giardial drugs or a vaccine to control giardiasis.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ESV, encystation-specific vesicle
CWP, cyst wall protein
GlcCer, glucosylceramide
GlcT1, glucosylceramide transferase 1
gGlcT1, giardial glucosylceramide transferase-1
GCS, glucosylceramide synthase
SL, sphingolipid
GM1, mono-sialotetrahexosylceramide
GM3, mono-sialodihexosylceramide
GD3, di-sialodihexosylceramide
SPT, serine palmitoyltransferase
PPMP, D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol
PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
NBD, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)] Bodipy, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
PC, phosphatidylcholine
PG, phosphatidylglycerol
PE, phosphatidylethanolamine
SM, sphingomyelin
FDA, fluorescein diacetate
PI, propidium iodide
ESI, electrospray ionization
GSL, glycosphingolipid
ER, endoplasmic reticulum
gPIS, PI Synthase/CDP-DAG-inositol 3-phosphatidyltransferase
gPGPS, PGP synthase/CDP-DAG-glycerol-3-phosphate-3-phosphatidyltransferase
gPSD, PS decarboxylase
gPSS, PS synthase
gPLTATPase 1A, Phospholipid-transporting ATPase IA
gLPAAT, Lysophosphatidic acid acyltransferase
gFAELO, Fatty acid elongase 1
gASMase, Acid sphingomyelinase-like phosphodiesterase 3b
gSPT1, Serine palmitoyltransferase-1
gSPT2, Serine palmitoyltransferase-2
gPI3K, Phosphoinositide-3-kinase, class 3

Chapter 1: Introduction

Giardia lamblia is an intestinal protozoan that causes giardiasis, a waterborne illness throughout the world including the U.S. The symptoms of giardiasis are diarrhea, fatigue, vomiting, weight loss and malabsorption. Diarrhea is considered the second major cause of death of children in developing countries (WHO Report, 2013). Parasitic protozoans make up a significant portion of cases of diarrhea and *Giardia* has contributed to 35.2% of all protozoan outbreaks from 2004-2010 (Lane et al. 2002; Baldursson et al. 2011). According to the Central for Disease Control and Prevention (CDC) report, there were 19000 cases of giardiasis between 2010-2012 in the U.S. [CDC report (2012) and (Yoder et al. 2010)]. *Giardia* has eight different assemblages (A-H) and human infections being consolidated within assemblage A and B (Kulda J 1978; Mayrhofer et al. 1995; Lasek-Nesselquist et al. 2010). Also, there have been cases of cross-infections between species such as the one between humans and dogs. Dogs are infected by assemblages C and D however, one dog was recorded to have both B and C infections (Hopkins et al. 1997). Moreover, small portions dairy cattle have been shown to be infected with assemblage A (<20%) in Canada and Australia (O'Handley et al. 2000; Applebee 2002). Besides dogs and cattle, many animals can serve as reservoirs for humans.

Giardiasis can be symptomatic and non-symptomatic. Sixty percent of all infections worldwide are asymptomatic where children are mostly affected (Ortega et al. 1997; ECDPC/ECDC, 2008). Severe and chronic giardiasis have been shown to cause irritable bowel syndrome, fatigue and in rare cases arthritis, cholecystitis, and pancreatitis even after the infection has cleared (Wolfe 1992). In children, chronic giardial infection has been shown to cause urticaria, low IQ and stunted growth (Sackey et al. 2003; Wright 2012). Incidentally, no pathogenic marker has been identified in *Giardia* and no suitable animal model to study giardiasis was developed.

Giardia has two morphologic forms: The metabolically active and vibrant trophozoites, and a relatively dormant cyst (**Fig 1**). The infective cyst is transmitted through fecal contaminated water or food, and it is estimated that 10 cysts or less are needed to establish an infection (Talal et al. 1994). When cysts are ingested, the cyst-wall lining is degraded by the low pH environment of the stomach to

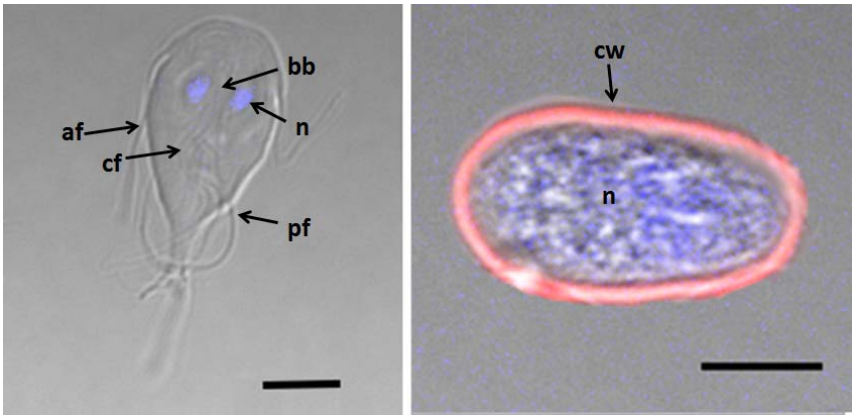

Figure 1: Morphology of *Giardia*

The giardial trophoizoite (active stage) and the cyst (dormant stage). The active trophozoite stage contains two nuclei (n) with a set of basal bodies (bb) between them. For movement the parasite relies on anterior (af), caudal (cf) and posterior flagella (pf). The dormant cyst contains four nuclei (n) enclosed within a cyst wall (cw).

begin excystation. Upon its release into the duodenum, *Giardia* cyst differentiates into two trophozoites and begins colonizing the small intestinal lumen where it remains non-invasive and multiplies by asexual reproduction (binary fission). Further down in the small intestine, *Giardia* undergoes encystation or cyst formation (Adam 2001).

Giardia is a binucleate unicellular parasite that is able to survive outside in the environment by forming a cyst. Designated as an early-divergent eukaryote, *Giardia* maintains a unique system which is comprised of both eukaryotic and prokaryotic characteristics (Morrison et al. 2007). It has no classical Golgi apparatus, but secretes Golgi-like vesicles called Encystation-Specific Vesicles (ESVs) during the cyst formation (Reiner et al. 1990; Stefanic et al. 2009). Although *Giardia* lacks mitochondrion, it synthesizes an incomplete version of mitochondrion called a "mitosome" (Regoes et al. 2005). Interestingly, this "mitosome" is not known to carry out any of the mitochondrial functions and the significance of this incomplete/non-functional organism is not clear at this point. Instead of lysosomes, this parasite contains peripheral vacuoles (PV) that act as an endosome-lysosome fusion vesicle and store cathepsin B-like protease enzyme (Ward et al. 1997). These PVs fuse with the cell membrane to allow extracellular materials to be endocytosed (Touz et al. 2003). The cyst contains four nuclei of which two trophozoites will emerge during excystation. However, during encystation or differentiation into cyst stage (discussed below), *Giardia* exhibits polyploidy and replicates its genome from 4N → 8N → 16N (Poxleitner et al. 2008; Reiner et al. 2008; Carpenter et al. 2012).

1.1 *GIARDIA*: MOLECULAR MECHANISMS OF ENCYSTATION AND EXCYSTATION

While the exact mechanism that trigger encystation and excystation are not well understood, it has been proposed that exposing cells to conditions that most closely resemble those within the host are most effective in producing trophozoites and cysts (Bingham et al. 1979). In the host, cysts are exposed to highly acidic conditions as they pass through the stomach, followed by a quick neutralization once in the duodenum. In the small intestine, trophozoites are exposed to bile salts and digestive enzymes with detergent-like activity. In the jejunum, excystation takes place and *Giardia* comes in contact with lactic

acid produced by bacteria present in the small intestine (**Fig 2**). Furthermore, it has been shown that exposure to low pH and pancreatic proteases is crucial for the excystation process (Boucher et al. 1990). An excyzoite (i. e, a cyst in the process of excystation) divides twice to produce four trophozoites (Bernander et al. 2001).

When encystation is initiated in *Giardia*, the flagella are internalized and the ventral disk is deconstructed, resulting in the parasite detaching from the small intestinal lumen (Lauwaet et al. 2007). Cyst Wall Proteins (CWPs) 1, 2 and 3 begin to be synthesized for transport toward the plasma membranes of trophozoites. The production of CWPs have been shown to be regulated by sterol regulatory-element-binding proteins (SREBPs), in which many proteins can be post-translationally modified by the intermediates of cholesterol metabolism (Lujan et al. 1995; Worgall et al. 2004).The CWPs are transported by the ESVs (**Fig 3**), ultimately fusing with PVs before laying down the cyst wall (Lauwaet et al. 2007). Giardial cyst wall is comprised of 40% protein including high-cysteine non-variant cyst protein (HCNCp), giardial Myb2, cysteine-protease-2, novel cyst proteins with epidermal growth factor repeats and Rab 11 (DuBois et al. 2008; Huang et al. 2008; Castillo-Romero et al. 2010). The remainder of the cyst wall is composed of lipids and carbohydrates. *N*-acetylgalactosamine (GalNAc) is the main carbohydrate found in the cyst wall and with all of these components, *Giardia* is able to withstand the oxidative environment in the cyst form (Gerwig et al. 2002). Cumulative reports suggest that *Giardia* has evolved a well-regulated secretory pathway to transport organelles and vesicles during encystation and excystation (Faso et al. 2011). During encystation, in the small intestine, trophozoites synthesize ESVs that are necessary to transport CWPs and other cyst-wall materials to the plasma membrane to form the cyst wall. The biogenesis of ESVs take place in the endoplasmic reticulum (ER), located in the perinuclear membranes in early-encystation cells, and then distributed extensively in the periphery of the cell (in close proximity to peripheral vacuoles) in late encystation phase (McCaffery et al. 1994). Presumably, the first half of the encystation phase is involved in triggering the encystation-specific genes, proteins and enzymes that are packaged into newly synthesized ESVs. Although, *Giardia* has no Golgi, it appears that ER participates in Golgi-like sorting and ESV itself shows Golgi-like appearance (Reiner et al. 1990; Stefanic et al. 2009). Thus like a

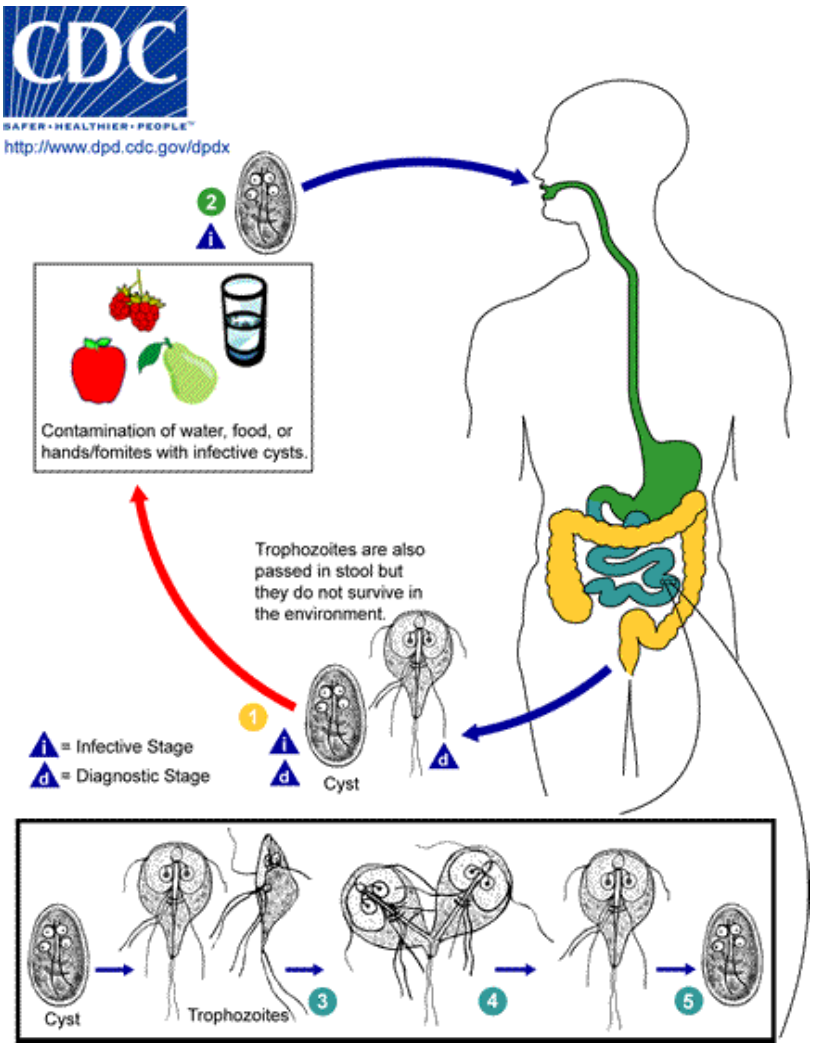

Figure 2: Lifecycle of *Giardia lamblia*

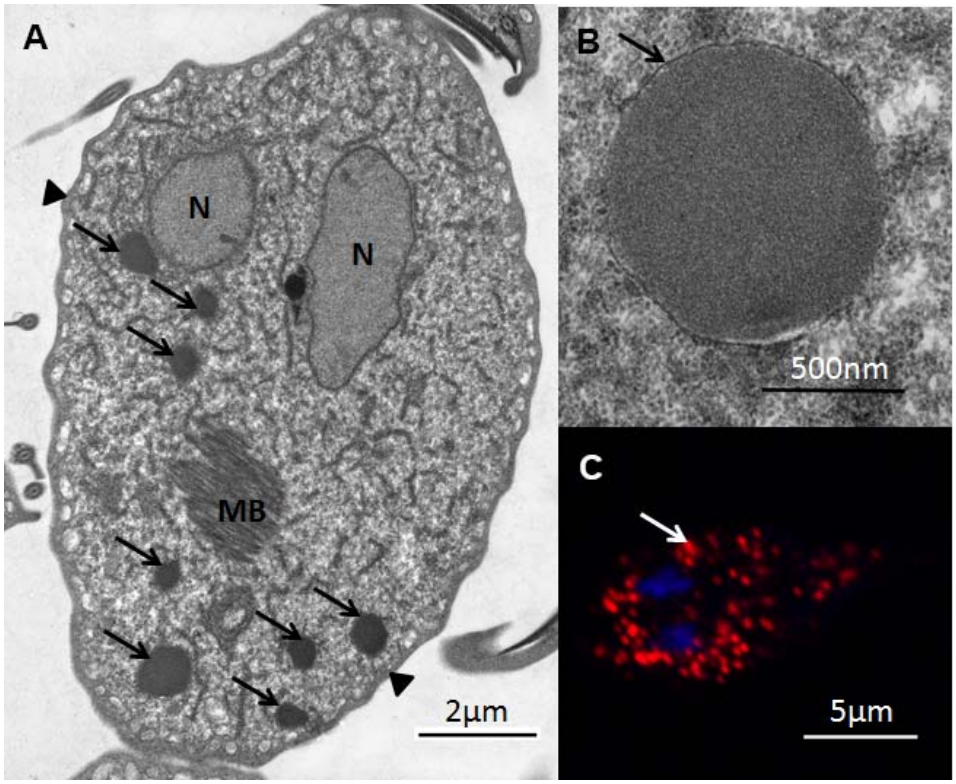

Figure 3: Formation of ESVs during encystation

Lower magnification (A) and higher magnification (B) TEM sections stained with "tannic acid" showing ESVs (arrow) during encystation. The ESVs head toward the plasma membrane (arrowhead). Nuclei (N) and median body (MB) are present. The confocal image (C) was treated with giardial cyst antibody to mark the ESVs (arrow) during encystation.

Golgi apparatus, ESVs contain Golgi-like cisternae that are involved in transporting cyst-wall components to plasma membranes (Faso et al. 2011). This postulation can be further supported by the fact that the ESV formation and secretion process in encysting *Giardia* is dependent upon COPII and small GTPase Rab 1, both markers for Golgi activity (Stefanic et al. 2009). Excystation, unlike encystation is somewhat simpler. During the passing of cysts through an acidic environment in the stomach, a cathepsin-B like protease (CP2) secreted from PVs degrades CWPs and at the same time parasite-specific glycohydrolase cleaves GalNAc fibrils of the cyst wall (Chatterjee et al. 2010). Secretion of CP2 into the inter-cyst wall space is triggered during excystation. Furthermore, the dephosphorylation of cyst wall proteins by secreted lysosomal acid phosphatases is critical to trigger excystation by *Giardia* (Slavin et al. 2002).

1.2 *GIARDIA*-LIPID INTERACTIONS IN THE HUMAN SMALL INTESTINE

Metabolism in *Giardia* is mainly processed through carbohydrate-derived energy. The conversion of glucose to pyruvate occurs through Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways and unlike higher eukaryotes, it is not compartmentalized (Lindmark 1980; Adam 2001). Another metabolic pathway in this parasite is the metabolism of alanine, aspartate, and arginine amino acids (Mendis et al. 1992; Schofield et al. 1992; Schofield et al. 1995). *Giardia* as a microaerophile, produces ATP by converting arginine to orthinine (substrate level phosphorylation) without the presence of oxygen. Moreover, arginine can be catabolized to citrulline by arginine deaminase, which yields 7-8 more ATPs more than glucose (Schofield et al. 1992). Other than glucose and arginine, intestinal lipids also play an important role in giardial biology. During their colonization in the human small intestine, *Giardia* is exposed to bile salts and dietary fats. Reports suggest that intestinal fatty acids kill *Giardia*, whereas mucus and bile salts protect the parasite from being killed by fatty acids and other small intestinal factors (Reiner et al. 1986; Das et al. 1988). Bile acids were also proposed to facilitate the transport of intestinal lipids into *Giardia* by forming mixed micelles (Das et al. 1997). Free fatty acids generated from phospholipids and triglycerides are inhibitory to the growth of *Giardia* (Reiner et al. 1986; Das et al. 1988). Studies suggest that dodecanoic ($C_{12:0}$) acid (also known as

lauric acid) possesses an anti-giardial property at a reasonably low concentration (Rayan et al. 2005). This medium-chain fatty acid accumulates inside trophozoites and alters membrane permeability and integrity. *Giardia* has the machinery to neutralize the toxic effects of free fatty acids by forming complex with membrane proteins, lipids, and carbohydrates (Das et al. 1991; Gibson et al. 1999; Touz et al. 2005). However, Gillin et al. (1987; 1988) have demonstrated that at lower concentrations, fatty acids induce encystation. Subsequently, cholesterol and bovine bile, which *Giardia* obtains from the growth medium, were shown to induce encystation (Kane et al. 1991; Lujan et al. 1996). Interestingly, the homologues of sterol regulatory-element-binding proteins (SREBPs) were identified in *Giardia* and found to regulate the expression of CWP genes during encystation (Worgall et al. 2004). Several proteins of the parasite can undergo post-translational modification by the intermediate of cholesterol (isoprenyl-group) biosynthetic pathway (Lujan et al. 1995) and it is possible that these modifications of giardial proteins are important for maintaining membrane integrity and functions.

1.3 AN ORGANISM WITH MINIMAL LIPID SYNTHESIS ABILITY

Since *Giardia* has a limited capability to synthesize lipid molecules de novo and as mentioned above, the majority of lipids are obtained from the small intestinal environment and remodeled accordingly (Das et al. 2001). Using radioactive acetate, glucose, glycerol, threonine, cholesterol and glycerol-3-phosphate, Jarroll and his colleagues (1981) monitored the metabolic conversions of these lipids into other lipid products. Surprisingly, it was noted that none of these radioactive precursors were incorporated into other lipids. This proposal was further supported by Kaneda and Goutsu (1988) and Mohareb *et al.* (1991), who showed that the lipid composition in *Giardia* is similar to that of the growth medium. Results suggested that phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and phosphatidylglycerol (PG) are present in both trophozoites and encysting cells and do not change during encystation (Ellis et al. 1996). Our mass spectrometric results indicated that PCs and PGs are the major phospholipids in *Giardia*. Seventeen different species of PGs are present with various combinations of odd- and even-numbered, carbon-containing fatty acids. Relative quantitation of individual lipids further elucidated that two PG species containing $C_{18:1}/C_{16:0}$ and

$C_{18:1}/C_{16:0}$ were most abundant, followed by $C_{16:0}/C_{16:0}$ and/or $C_{18:0}/C_{14:0}$. Nineteen species of PCs were detected and among them C18:1/C18:1 was found to be the major PC. In addition to PGs and PCs, six species of PEs, three species of SMs and two species of phosphatidylinositols (PIs) were also detected (Yichoy et al. 2009). Interestingly, except for lyso-PCs and PCs, no other phospholipids are present in bile and serum, suggesting that many of these phospholipids (specifically PG and PE) in *Giardia* could be synthesized de novo via CDP-DAG and/or fatty acid and head-group remodelling pathways (Das et al. 2001). This hypothesis can be further supported by the fact that radiolabeled fatty acids are directly incorporated into giardial phospholipids (Blair et al. 1987; Stevens et al. 1997; Gibson et al. 1999; Subramanian et al. 2000; Vargas-Villarreal et al. 2007), indicating that *Giardia* has the enzymes and pathways to synthesize new phospholipids most likely by fatty acid remodelling reaction which is also known as the Lands cycle (Das et al. 2001). Radiolabeled bases (i.e., choline, inositol, ethanolamine, serine and glycerol) are also incorporated into respective phospholipids of trophozoites when added to the culture medium (Subramanian et al. 2000); Das *et al*. unpublished), suggesting that like fatty acid remodelling reactions, base exchange or headgroup-exchange reactions are operative in this parasite suggesting that *Giardia* relies more on remodelling pathways rather than the de novo synthesis via CDP-diacylglycerol pathway.

1.4 STORIES TOLD BY THE LIPID GENES

The curiosity that *Giardia* has a limited lipid synthesis ability prompted us to analyze the lipid and fatty acid genes that are annotated in the *Giardia* Genome Database (www.GiardiaDB.Org; (Morrison et al. 2007). Nine genes of phospholipid synthesis, transport and metabolism were identified in *Giardia*, including the putative homologues of PC synthase (gPCS), phosphatidylinositol synthase (gPIS), PI transfer protein (alpha isoforms, gPITPα), phosphatidylglycerolphosphate synthase (gPGPS), phospholipid-transport (gPLT) ATPase 1A and IIB, phosphatidylserine synthase (gPSS), phosphatidylserine decarboxylase (gPSD) and headgroup (choline/ethanolamine) kinases. The presence of phospholipid-transport ATPase IA and IIB (also known as phospholipid flippases or gFLIP) suggest that *Giardia* has evolved an efficient mechanism to internalize phospholipid molecules, especially PC

from its small intestinal environment. Among various phospholipid genes, the presence of gPIS, gPGPS, gPSS and gPSD are also interesting and may signify the capability of *Giardia* to synthesize limited phospholipids de novo. Earlier, we have demonstrated that genes for gPGPS and gPSD are expressed in *Giardia* and remain unchanged throughout the life-cycle of the parasite, suggesting that they may function as a house-keeping gene (Yichoy et al. 2009).

The *Giardia* genome project predicts the presence of nine fatty-acid (FA) transport, synthesis, and metabolic genes. Three 1-acyl-sn-glycerol-3-phosphate acyltransferases (SLCs)—i.e., gSLC2, gSLC3 and gSLC4 —were annotated in the database, suggesting that *Giardia* might use these gene products to import fatty acids from its environment. Other FA genes annotated are putative lysophosphatidic acid acyltransferase (gLAAT), elongase 1 (gELO) several long-chain fatty-acid (LCFA)-CoA ligases (gLCFA-CoA ligases) [i.e., LCFA-CoA ligase 4 (gLCFLA4*)* and three different forms of LCFA-CoA ligase 5 (gLCFLA5)] and acetyl-CoA/pyruvate carboxylase (gACPC). The presence of these FA genes further indicates that very basic and essential FA metabolism can be carried out by *Giardia,* such as transferring fatty acids across the membranes, forming reactive FA species (fatty acyl-CoA), acylating lysophosphatidic acid (LPA) to form phosphatidic acid (PA), and elongating and ligating FA chains (Table 2). *Giardia* contains two isoforms of secreted and cytoplasmic phospholipase B enzymes (*gplb*) that are responsible for removing *Sn1* and *Sn2* fatty acids from a phospholipid at the same time (Morgan et al. 2004).

Only five SL metabolic genes were annotated in the *Giardia* database, including the genes that encode SPT 1 & 2, GlcT-1 (*gglct1*), and two separate ASMases enzymes (ASMase B [gasmase b] and ASMase 3b [*gasmase 3b*]). It has been reported earlier that all of these five genes are expressed and regulated differentially in two different stages of the life cycle of *Giardia*, suggesting that SL pathways could be involved in modulating the growth and differentiation of this waterborne pathogen (Hernandez et al. 2008). Various lipid kinase genes were also annotated. These include the genes for putative target of rapamycin (gTOR), PI-3, -4, -5-trisphosphate 3-phosphatase (gPIPase), inositol-1, -4, -5-trisphosphate 5-phosphatase (gITPase), inositol 5-phosphatase PI-3-kinase class 3 (gPIK), alpha polypeptide of PI-3-kinase catalytic subunit (gPI3K), three isoforms of PI-4-phosphate 5-kinase

(gPIPase), PI-4-kinase (gPI4K), and PI-glycan biosynthesis, class O protein (gPIG). The molecular and bioinformatic analyses revealed that giardial TOR is an analogue of the FRAP/TOR of eukaryotes expressed in dividing parasites and may not be inhibited by rapamycin (Morrison et al. 2002). The bioinformatic analyses of three giardial PIKs genes (gPIK*s*)—two gIPKs (gPI3K-1 and gPI3K-*2*) and one gPI4K—were also studied (Cox et al. 2006; Hernandez et al. 2007). The identified genes contain catalytic (p110) but not regulatory (p85) subunits. Transcriptional analyses demonstrated that *gpiks* are expressed in *Giardia* and modulated during encystation. In addition, two PI3K inhibitors, wortmannin and LY 294002, inhibited the replication of trophozoites in culture, supporting the notion that PIKs' activities could be linked to the growth and encystation of *Giardia*.

1.5 SPHINGOLIPIDS AND ENCYSTATION

Sphingolipids (SLs) are sphingosine-based phospholipids that control various biological mechanisms such as membrane biosynthesis, cell to cell communication, signal transduction and apoptosis (Hanada 2005). Our lab has extensively studied the SL pathway in *Giardia* and found that this parasite has a limited SL synthesis capability. As mentioned above, only five SL genes are present in *Giardia* and they are: serine palmitoyltransferase-1 (*gspt-1*), serine palmitoyltransferase (*gspt-2*), ceramide-glucosyltranferase-1 (*gglct-1*), sphingomyelinase b (smase b), and sphingomyelinase 3b (*smase 3b*). It is clear that there are gaps within the SL pathway in this parasite, yet each portion of the pathway seems to have a different role in its lifecycle. All five genes are differentially regulated during encystation and giardial serine palmitoyltransferase enzyme (gSPT) is responsible for endocytosis. On the other hand, giardial glucosyltransferase enzyme (gGlcT1) has been shown to partake in encystation, whereas the function of another abundant SL-hydrolyzing enzyme, sphingomyelinase (gSMase) is still not clear (Hernandez et al. 2008).

1.6 THE KNOWLEDGE GAP AND THE FOCUS OF THE CURRENT DISSERTATION

Giardia is a non-invasive parasite, and the mechanism by which it causes disease is not clear. No known toxin or virulence factor is secreted by this parasite but the ability of *Giardia* to form cysts could be considered its "virulence determinant" because the formation of viable cysts allows this organism to survive in a harsh environment and to spread the infection. The first step of cyst formation is the synthesis of encystation-specific vesicles (ESVs) involved in transporting cyst-wall materials that later fuse with the plasma membrane and lay down the cyst-wall. Three encystation-specific cyst-wall proteins (CWP-1, -2, and -3) are expressed at the time of encystation and concentrated within the ESVs (Lauwaet et al. 2007). Biochemical analyses demonstrate that cyst-wall of *Giardia* contains insoluble filamentous materials that consist of glycoprotein and amino-sugar containing polysaccharides (Gerwig et al. 2002; Sener et al. 2004) Despite all this information, the mechanism of encystation and cyst formation is not well understood. Earlier experiments conducted in our laboratory indicated that SLs are important for giardial encystation. L-cycloserine, an inhibitor of SPT was shown to block endocytosis and endoplasmic reticulum/perinuclear targeting of Bodipy-conjugated ceramide in trophozoites which could be reversed by 3-ketosphinganine, a product of this enzyme. Likewise, D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), an inhibitor of glycosylceramide (GlcCer) synthesis, blocked karyokinesis and reduced the cyst production in culture (Hernandez et al. 2008). It has also been reported that PPMP inhibits trophozoite replication and produces incompletely divided cells (doublets or triplets) and thereby block encystation (Sonda et al. 2008; Stefanic et al. 2010). However, none of these reports shed any light on the mechanism by which SLs, especially the enzyme, gGlcT1 regulates encystation and cyst production by this parasite.

My dissertation focuses on understanding the unique and precise function of gGlcT1 and its role in encystation and cyst formation. I found that modulation of gGlcT1 activity by overexpression and knockdown affects the ESV biogenesis and cyst viability of *Giardia*. I also show that gGlcT1 overexpression disturbs lipid homeostasis in this parasite by elevating the intracellular levels of lipids, cholesterol, and fatty acids without affecting the membrane fluidity. Finally, a model depicting the

current results outline how the gGlcT1 enzyme may function during encystation. With this information gGlcT1 can be targeted for developing new anti-giardial therapy.

.

Chapter 2: Glucosylceramide Transferase Regulates Encystation and Cyst Production by *Giardia lamblia*

The synthesis of glucosylceramide (GlcCer; a glycosphingolipid) is catalyzed by the glucosylceramide transferase-1 (GlcT-1) enzyme that transfers glucose from UDP-glucose to ceramides as shown below.

$$\text{UDP-Glucose + Ceramide} \xrightarrow{\text{GlcT1}} \text{Glucosylceramide + UDP}$$

Interestingly, ceramide, like many other lipids, is not synthesized de novo by this parasite. Our laboratory has reported earlier that ceramide (an important lipid of plasma and endomembranes) is taken up by *Giardia* from the cell exterior via the clathrin-dependent pathway and targeted to the endoplasmic reticulum (ER)/perinuclear membranes (Hernandez et al. 2007). Studies also suggest that the GlcT1 enzyme in *Giardia* is embedded in the ER membranes where GlcCer synthesis apparently takes place (Hernandez et al. 2008). Newly synthesized GlcCer then participates in encystation and cyst production (Hernandez et al. 2008; Sonda et al. 2008; Stefanic et al. 2010). The conclusion—i.e., that GlcCer is important for encystation stemmed from two different observations: (1) that the transcript of the *gglct1* gene is upregulated during encystation, and (2) that D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PPMP), an inhibitor of GlcCer synthesis, blocks encystation and cyst production by *Giardia* in culture. However, what is not known is whether the *gglct1* gene encodes the active gGlcT1enzyme, and whether PPMP blocks encystation by inhibiting gGlcT1. The BLAST analysis reveals that the gGlcT-1 sequence is highly divergent from other organisms, with *Arabidopsis thaliana* being the closest relative with 32% amino acid identity. In addition, the catalytic domain gGlcT1 has yet to be elucidated. Further study of gGlcT1 may help us in discovering the structural components and catalytic site of this enzyme. The lack of similarity of gGlcT1 to other organisms may also help in finding a unique drug target against this parasite.

In other organisms, GlcT1 has been implicated in several functions. The overexpression of GlcT1 (also known as glucosylceramide synthase or GS) causes multi-drug resistance in breast cancer cells and through the use of RNAi methodology this drug-resistant effect could be reversed (Sun et al. 2006). In diet-induced obesity in mice, the inhibition of GlcT1 decreased insulin resistance, whereas the GlcCer synthase inhibitor Genz-123346 reversed cystogenesis in mice with autosomal polycystic kidney

disease (Natoli et al. 2010; Yew et al. 2010). In terms of cell structure of *Cryptococcus neoformans*, GlcCer is essential for cell-wall formation, cell growth, and budding, and *Candida albicans* and *Pichia pastoris* with a GlcT1 deletion are resistant to RsAFP2, an antifungal plant defensin (Thevissen et al. 2004). In addition, in *A. thaliana* the inhibition of GlcCer synthase with PPMP disrupts Golgi morphology and protein secretion (Melser et al. 2010).

The experiments described in Chapter 2 were carried out to identify the mechanism by which GlcT1 regulates encystation and cyst production. It was mentioned in Chapter 1 (Introduction) that during encystation ESVs are packed with CWPs that are synthesized in the ER. The ESVs are formed directly from the ER, which in the case of *Giardia* is found throughout the cytoplasm, occasionally connecting the plasma membranes to the PVs (Faso et al. 2011). I have used molecular methodologies (i.e., overexpression, knockdown, and rescue) to modulate gGlcT1 activity and have evaluated the biogenesis of ESVs and cyst production by this waterborne pathogen. I have also investigated whether gGlcT1 activity is critical for maintaining the cyst viability because infections (giardiasis) are caused when viable cysts are ingested by humans.

2.1 MATERIALS AND METHODS

2.1.1 Materials

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and were of the highest available purity. Tetramethyl rhodamine (TMR)-conjugated goat anti-mouse antibodies; fluorescent (NBD-conjugated) lipids; 4' and 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA). D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), and other lipid standards including [^{13}C]-deuterated GM3 were purchased from Matreya LLC (Pleasant Gap, PA). Glucosylceramide and lactosylceramide standards were obtained from Avanti Polar Lipids (Albester, AL). Anti-giardial cyst and anti-AU1 antibodies (monoclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Covance Laboratory (San Diego, CA), respectively. UDP-[^{14}C] glucose (300 mCi/mmol) was purchased from American Radiolabeled Chemical (St. Louis, MO).

2.1.2 Trophozoites, encysting cells, and the generation of in vitro cysts

Giardia lamblia trophozoites (strain WB, ATCC No. 30957) were cultivated following the method of Diamond et al (1978) using modified TYI-S-33 medium supplemented with 5% adult bovine serum and 1% bovine bile (Keister 1983). The antibiotic piperacillin (100 μg/ml) was added during routine culturing of the parasite (Gillin et al. 1989). Trophozoites were detached from the culture flask by ice chilling and harvested by centrifugation at 1,500 x g for 10 min at 4 °C, washed with PBS and microscopic determination of cell numbers using a hemocytometer. In vitro encystation was carried out by culturing the trophozoites in TYI-S-33 medium (pH 7.8) supplemented with adult bovine serum (10%, v/v), lactic acid (5 mM), and porcine bile (250 mg/ml) for various time points, as described below (Gillin et al. 1989). Cells were allowed to encyst for 72 h, and cysts were isolated by centrifugation (2,500 x g for 10 min at 4 ^{o}C); washed in distilled water, and kept in water for three days at 4^{o}C. Isolated water-resistant cysts were counted or subjected to the microscopic experiments also described below.

2.1.3 Overexpression of gGlcT1 in *Giardia* trophozoites

For overexpression, a small peptide-epitope (AU1)-tagged pNT5 expression plasmid (obtained from Dr. Chin-Hung Sun, Taiwan) containing *gglct1* gene was constructed following the method described by Pan et al. (Pan et al. 2009). The entire open-reading frame of *gglct1* ORF_11642 was amplified by PCR using the primers 5‘-GCGCCATGGATGGACGGGTTGACTCTCTCC-3‘ and 5‘-GCGGAATTCTCAGATGTATCGATACGTATCGTCGAGGGATTTTTT-3‘. The insert was digested with EcoR1/Nco1 and ligated into EcoR1/Nco1-digested dephosphorylated pNT5 plasmid (Pan et al. 2009). This new plasmid pNT5-*gglct1* was then transformed into competent DH5α cells and plated onto LB plates containing 100μg/ml ampicillin. Colonies were screened by PCR and positive colonies were sequenced at the University of Texas-El Paso (UTEP) DNA core facility. The ABI Prism BigDye Terminator v3.1 Cycling Sequencing Kit (Applied Biosystems, Carlsbad, CA) was used to amplify the DNA with fluorescently labeled dideoxynucleotides. The sequencing reaction was subsequently cleaned using Agencourt Clean SEQ (Beckman Coulter, Brea, CA). The parasites were placed in a 4-mm electroporation tube (Fisher Biotech, Waltham, MA) in a 300-μl suspension of TYI-SS media where 40

μg of the pNT5-*gglct1* plasmid was added. Trophozoites were then transfected by electroporation on a BioRad Gene Pulser X cell (BioRad, Hercules, CA) using the following parameters: 322 V, 500 μF, and ∞Ω resistance (4-mm cuvette). Cells were allowed to recuperate in media overnight, followed by selection with 150 μg/ml G418 (Sigma Aldrich, St. Louis, MO). Stable transfectant trophozoites overexpressing gGlcT1 (+gGlcT1) were established within two weeks.

2.1.4 Knockdown of gGlcT1 expression by anti-sense morpholino oligonucleotide

Cultured *Giardia* trophozoites were harvested by centrifugation as described above. Parasites were placed in a 4-mm electroporation tube (Fisher Biotech) in a 300-μl suspension of the media, transfected (using the same setting as the overexpression described above) with anti-GlcT-1 (5'-CTAAGGAGAGAGTCAACCCGTCCAT-3'), and scrambled (mismatch) with (5'-CCTCTTACCTCAGTTACAATTTATA-3') morpholino oligonucleotides (Gene Tools, Inc., Philomath, OR) following the protocol described by Carpenter and Candi (2009). gGlcT1 knockdown trophozoites were resuspended in media and left to recover for 6 h for further experimentation.

2.1.5 Assessing PPMP and morpholino oligonucleotide-treated trophozoites

Giardial trophozoites were electroporated with 50 μM anti-*glct1* morpholino and/or treated with PPMP (10 μM) for 24 h. Cells were then harvested and mounted with Prolong® Gold Antifade Reagent (Invitrogen) in a four-well chambered slide as described above without any further treatments. At least 100 singlet, doublet and triplet trophozoites from 10 different fields were viewed and counted by confocal microscopy (Zeiss-LSM 700) using Zen 2009 software (Carl Zeiss, Irvine, CA). The percentage values of doublet cells were calculated based on the total number cells (singlet, doublet and triplet).

2.1.6 Rescue experiments.

The stable transfectants of *Giardia* trophozoites overexpressing gGlcT1 were generated as described above. gGlcT1-overexpressing trophozoites were then transfected with anti-gGlcT1 morpholino oligonucleotide following the procedure also described above. After anti-sense morpholino oligonucleotide transfection, gGlcT1-overexpressing trophozoites were allowed to recover for 6 h before conducting the experiments described below.

2.1.7 gGlcT1 assay

The activity of the gGlcT1 enzyme in *Giardia* trophozoites, encysting cells, cysts, gGlcT1-overexpressing, and -knockdown cells were measured using the methods described for mammalian and *Plasmodium* cells (Uchida et al. 2002; Couto et al. 2004). Each reaction tube contained phosphate buffer (100 mM, pH 7.4), crude enzyme extracts, UDP-[^{14}C]-glucose (~100, 000 cpm/assay), protease inhibitors (2.5% 1 x protease inhibitor cocktail, 5 μM E-64), 1 mM $MgCl_2$, 5 mM KCl, 2 mM β-NAD, and the liposomal substrate containing 0.1 nmol of ceramide mixed with dipalmitoylphosphatidylcholine (DPPC) in a ratio of 1:10 (v/v) (total volume, 100 μl). The mixture was incubated for 5 h at 37 °C and extracted with chloroform ($CHCl_3$): methanol (MeOH) 1:1 v/v), and the formation of enzymatic product ([^{14}C]-GlcCer) was assessed by measuring the radioactivity in a scintillation counter (Beckman, Brea, CA). The assay was conducted in the presence and absence of PPMP (10 μM) (Hernandez et al. 2008; Sonda et al. 2008; Stefanic et al. 2010).

2.1.8 Synthesis of NBD-C_6-glucosylceramide

NBD-GlcCer synthesis by *Giardia* was monitored following the method described by Gupta et al. (2010). Briefly, cells were harvested and resuspended in PBS supplemented with 1% glucose and 0.2% L-cysteine. A total of 50 μM of NBD-C_6 ceramide-BSA complex (Invitrogen) was added and incubated for 5 h at 37 °C. Sphingolipids were extracted in a solvent in a mixture containing 1.3 ml acetic acid: MeOH (1:50, v/v), 1.3 ml $CHCl_3$, and 1.3 ml of Milli-Q H_2O. The lower-$CHCl_3$ phase was

pooled and dried under nitrogen. Samples were resolved on TLC (Whatman, Waukesha, WI) using the following solution: 170 ml $CHCl_3$, 30 ml MeOH, and 2 ml 3.5 (N) ammonium hydroxide (NH_4OH). Formation of NBD-C_6-glucosylceramide was visualized under a UV lamp.

2.1.9 Staining with anti-cyst antibody for immunofluorescence

To evaluate the transition from non-encysting to encysting trophozoites and cysts as well as to identify ESVs, cells were labeled with the following antibodies: mouse anti-cyst antibody (1:100) (Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-GlcT1 custom antibody (1:200) (Thermofisher Scientific), TMR-conjugated goat anti-mouse antibody (1:500) (Invitrogen), Fluorescein-conjugated goat anti-mouse antibody (1:500) and goat anti-rabbit Alexa Fluor 594 (1:500). The anti-cyst antibody was raised against intact cysts and therefore recognizes all cyst wall proteins (CWP1, CWP2 and CWP3). Trophozoites were cultivated and subjected to encystation in a cultured medium as described before (Gillin et al. 1989). Cells were harvested and fixed to four-chambered slides using 4% paraformaldehyde. Fixed samples were allowed to react with anti-cyst and anti-GlcT1 antibody overnight at 6–10^{o}C, followed by reacting with corresponding secondary antibody for 2 h at room temperature. Cells were subsequently mounted with Prolong® Gold Antifade Reagent mixed with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen). Samples were analyzed in a confocal microscope (Carl Zeiss Laser Scanning Systems LSM 700), using the Zen 2009 software (Carl Zeiss) for acquisition and image analysis.

2.1.10 Glycosphingolipid analysis by ESI-MS-MS

Neutral and acidic glycosphingolipids (GSLs) were extracted and separated following the method described by Li et al (2008). Briefly, control, gGlcT1-overexpressed, -knockdown and -rescued trophozoites (~1x10^{9} cells/sample) were harvested, pelleted, and extracted first with $CHCl_3$: MeOH (1:1, v/v) and then with isopropanol: hexane:H_2O (55:25:20, v/v/v) before being subjected to DEAE-Sephadex chromatography. Neutral lipids were eluted with $CHCl_3$: MeOH: H_2O (30:60:8, v/v/v).

Acidic lipids were eluted with MeOH-containing 0.8 M sodium acetate. Both neutral and acidic GSLs were subjected to methylation as described by Ciucanu and Kerek (1984), followed by ESI-MS/MS analysis using an LTQ XL, Thermo Fisher Scientific). Briefly, samples were spiked with 1 pmol/µl methylated standards (d18:1/C12:0) and introduced via a TriVersa NanoMate nanoelectrospray source (Advion, Ithaca, NY) (capillary temperature 230 °C, with injection time of 100.00 ms, activation time of 30 ms, activation *Q*-value of 0.250, isolation width of *m*/*z* 2.0, and acquisition time of 0.5 min). Quantitation was achieved using select-reaction monitoring (SRM) with collision energy set to 50 for all samples. For hexosylceramide reactions, singly charged parent ions with sodium adduct ($[M+Na^+]^+$) were fragmented and monitored for the fragment ions of permethylated hexose $[C_{10}H_{20}O_6+Na]^+$ (from monohexosylceramide), permethylated dihexose $[C_{19}H_{36}O_{11}+Na^+]^+$ (from dihexosylceramide), or permethylated trihexose $[C_{28}H_{52}O_{16}+Na^+]^+$ (from trihexosylceramide). For the acidic GSLs (i.e., GM1, GM3, and GD3), the loss of methylated-sialic acid fragment at 376 Da was monitored $[M+Na^+]^+/[M+Na\text{-}C_{17}H_{30}NO_8]$. Hexosylceramide was normalized to the internal standard d18:1/C12:0-glucosylceramide (Avanti Polar Lipids), and dihexosylceramide and trihexosylceramide were normalized to the internal standard d18:1/C12:0-lactosylceramide (Avanti Polar Lipids). GM3, GD3, and GM1 were normalized to [^{13}C]-deuterated d18:1/C18:0-GM3 standard (obtained from Matreya). Due to differences in ionization efficiencies between individual lipids, relative abundance was calculated rather than absolute mass of each lipid class.

2.1.11 Monitoring cyst viability

Fluorescein diacetate (FDA) inclusion and propidium iodide (PI) exclusion experiments were performed to monitor cyst viability. Briefly, a 25-mM stock solution of FDA and PI (Sigma Aldrich) were prepared in 100 mM phosphate buffer (pH 6.0), from which a working solution was made by adding 40 µl of stock to 10 ml phosphate buffer. gGlcT1-overexpressing and -knockdown trophozoites were subjected to encystation as described above, and the water-resistant cysts were collected by centrifugation. Approximately, 10^7 cysts were subjected to FDA and PI staining as described previously by Gillin et al. (1989). Cysts were then washed with PBS and mounted on a slide with DAKO mounting

media (DAKO, Carpinteria, CA) and viewed under the Zeiss LSM 700 Confocal microscope using Zen 2009 software (Carl Zeiss).

2.1.12 Western blot analysis

Parasites were harvested and resuspended in lysis buffer (5mM Tris HCl; EDTA 2mM; Triton X-100; 0.1%; pH 7.4) containing protease inhibitor cocktail and E-64 (1 µM, Sigma). The sample was freeze thawed at -80 °C, boiled in sample buffer before being analyzed (40 µg protein/lane) by 10% SDS-PAGE, and was followed by immunoblotting analysis on the polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 1% BSA, incubated with anti-cyst antibody (monoclonal, 1:100) or anti-gGlcT1 antibody (polyclonal, 1:200) (Thermo Fisher Scientific) and incubated overnight at 4 °C. The PVDF membrane was then incubated with anti-mouse HRP conjugate (1:10,000; KPL, Inc., Washington, DC) or anti-rabbit HRP conjugate (1:5,000 [GlcT1]; KPL, Inc., Washington, DC) for 30 min at room temperature. The enhanced chemi-luminescence (ECL) technique was used for the detection of protein bands.

2.1.13 Statistical analysis

All values were given as mean values ± S.D., and statistical analyses were generated using SAS software ver. 9.2 (SAS Institute, Cary, NC) and Graphpad QuickCalcs (http://www.graphpad.com/quickcalcs/). In experiments with three or more variables, the F-test was performed, and in experiments with two variables the Student's *t*-test was performed. Statistical values less than 0.01 were considered highly significant, whereas values less than 0.05 were considered significant.

2.2 RESULTS

2.2.1 Giardial GlcT1 activity is stimulated at the time of encystation and reached maximum in mature cysts:

Earlier reports from this and other laboratories have indicated that GlcCer, a precursor for a wide variety of GSLs, plays an important role during encystation by *Giardia* (Hernandez et al. 2008; Sonda et al. 2008). It was observed that (i) the transcript of gGlcT1 gene (*gglct1)* is upregulated in encysting cells and (ii) PPMP, an inhibitor of GlcCer synthesis (Shukla et al. 1991; Kovacs et al. 2000), inhibits the cyst production in culture. In the current study, we asked if *Giardia* synthesizes the active gGlcT1 enzyme and uses it to drive the process of encystation. Therefore, gGlcT1 activity was measured in the cell-free extracts (1500 x g-supernatant) of non-encysting and encysting trophozoites as well as in water-resistant cysts using UDP-[^{14}C]-glucose and ceramide (Uchida et al. 2002; Couto et al. 2004). Results show that the basal gGlcT1 activity is low in non-encysting trophozoites and upregulated in 12- and 24-h encysting cells and increases several-fold in cysts (**Fig. 4*A***). It was found that the gGlcT1 activities in trophozoites and encysting cells were inhibited by PPMP which bears a resemblance to both ceramide and GlcCer (Shukla et al. 1991; Kovacs et al. 2000). However, unlike trophozoites and encysting cells, the cyst enzyme was completely resistant to PPMP (**Fig. 4*A***). Next, we generated GlcT1-knockdown *Giardia* where the *gglct1* gene was silenced by morpholino oligonucleotide. Knockdown by morpholino analogues is a faster and more efficient way to silence genes in *Giardia* (Carpenter et al. 2009). In recent years, various laboratories have successfully used anti-sense morpholino oligonucleotides to knockdown giardial kinesin and flagellar genes. The effects of anti-sense morpholinos are transient and last approximately 72 h, which is sufficient to carry out one round of the encystation cycle (Carpenter et al. 2009; House et al. 2011). As shown in **Fig. 4*B***, knockdown by anti-gGlcT1 morpholino reduced the gGlcT1 activity in cysts by ~60%. This result suggests that although resistant to PPMP, cyst gGlcT1 could be inhibited by anti-*gglct1* morpholino blocking the translation initiation of gGlcT1 by targeting the 5' region of the *gglct1* gene. On the other hand, PPMP competes with ceramide for the catalytic site of gGlcT1 and is thereby expected to inhibit the enzymatic reaction (Kovacs et al. 2000).

Synthesis of GlcCer, the reaction product of gGlcT1 was also monitored by assessing NBD-GlcCer synthesis by encysting cells. Both non-encysting and encysting *Giardia* trophozoites were labeled with NBD-C_6-ceramide, and the product was analyzed by TLC. **Fig. 4*C*** shows that the synthesis of NBD-GlcCer is extremely low (almost non-visible in the photograph) in trophozoites and increases during encystation. However, it was found that the formation of NBD-GlcCer in 12-h encysting cells is slightly higher than in 24-h encysting cells, which could be due to the fact that cells at the later stage of encystation (i.e., 18 h onwards) start forming cyst walls and become increasingly impermeable to NBD-ceramide and other molecules (data not shown). Therefore, no attempt was made to label the cyst with NBD-GlcCer. The increased synthesis of NBD-GlcCer during 12 h- and 24-h encysting *Giardia* supports our enzymatic results (**Fig. 4C**) that encystation stimuli induce gGlcT1 activity, leading to the synthesis of GlcCer.

Western Blot analyses with anti-gGlcT1 antibodies (custom ordered from Thermofisher) was performed to determine the protein expression levels during encystation (**Fig 5D**). The antibodies were observed on immunoblot and showed that GlcT1 labels proteins approximately ~30 and ~50 kD and the labeling increases as encystation progresses, which matches with the enzyme data (**Fig.4A**). When antibodies were pre-treated with their respective peptide antigens, no antibody-antigen reactions were observed, further demonstrating the specificity of the anti-gGlcT1 antibody.

Next, we proceeded with immunofluorescence of gGlcT1 custom antibody (Thermofisher Scientific). Giardial trophozoites, 24h encysting cells, and complete cysts were stained with both gGlcT1 and CWP antibodies. In trophozoites (**Fig 5A**), there is no CWP labeling but gGlcT1 forms vesicle-like aggregates that are cytoplasmic. In 24h encysting cells (**Fig 5B**), CWP antibodies label the ESVs that transport cyst wall material to the plasma membrane. On the other hand, gGlcT1 continues to label the vesicle-like structures, but they do not localize with the ESVs. Moreover, in encysting cells, gGlcT1 begins to localize between the nuclei where basal bodies and central mitosomes are located. When cyst wall is completely formed (**Fig 5C**), gGlcT1 is again cytoplasmic and shows no uniform localization pattern.

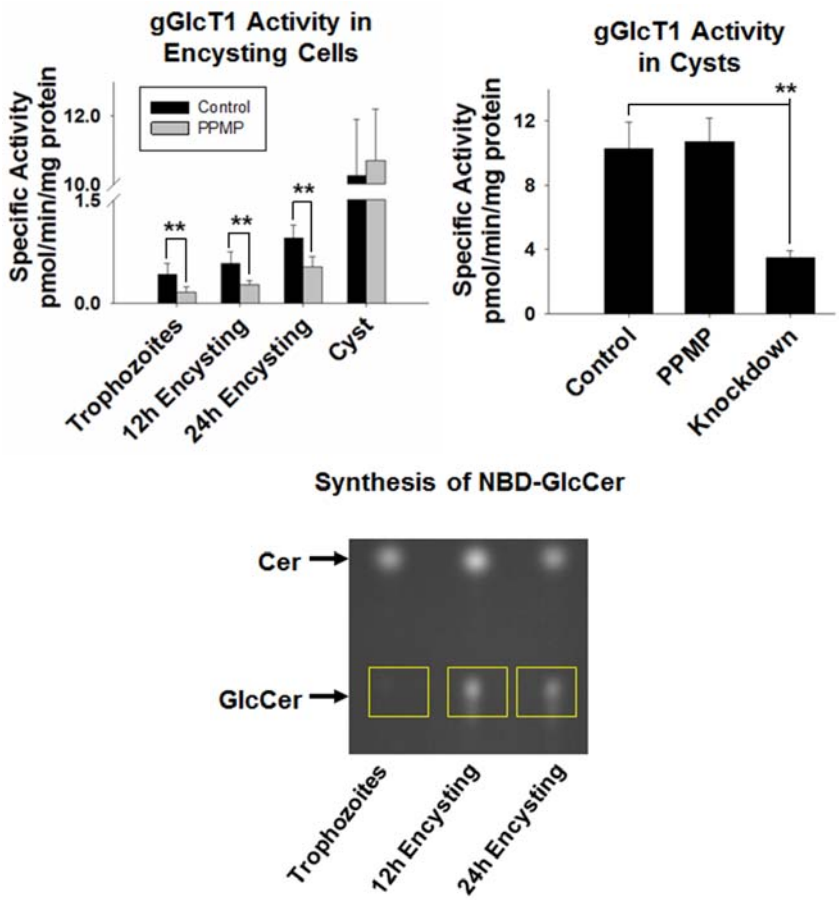

Figure 4: gGlcT1 activity in encysting *Giardia*.

A) gGlcT1 activity in nonencysting, encysting (12- and 24-h), and water-resistant cysts was measured in the cell-free extracts using [14C]UDP-glucose (100,000 cpm/assay) and ceramide as substrates. The levels of glycosphingolipids increase with encystation while PPMP inhibits its formation. Only in cysts is PPMP ineffective. Data represent means S.D. of three separate experiments, and the experiments were carried out in duplicate (**, $p < 0.01$). B) activity of gGlcT1 in control, PPMP-treated, and gGlcT1- knockdown cysts. Control and anti-gGlcT1 morpholino-transfected trophozoites were subjected to encystation. To assess the effect of the inhibitor, PPMP (10 μM) was added to the assay mixture. Again, although PPMP had no effect on glycosphingolipid formation in cysts, anti-gGlcT1 morpholino oligo suppressed the activity. The data represent means S.D. of three separate experiments (**, $p<0.01$). C) synthesis of NBD-GlcCer by encysting (12 and 24 h) trophozoites. Live cells were labeled with NBD-ceramide and sphingolipids were extracted and

analyzed by TLC. The data shows that GlcCer formation increases with encystation. The experiment was carried out three times, and the representative result from a single experiment is shown here.

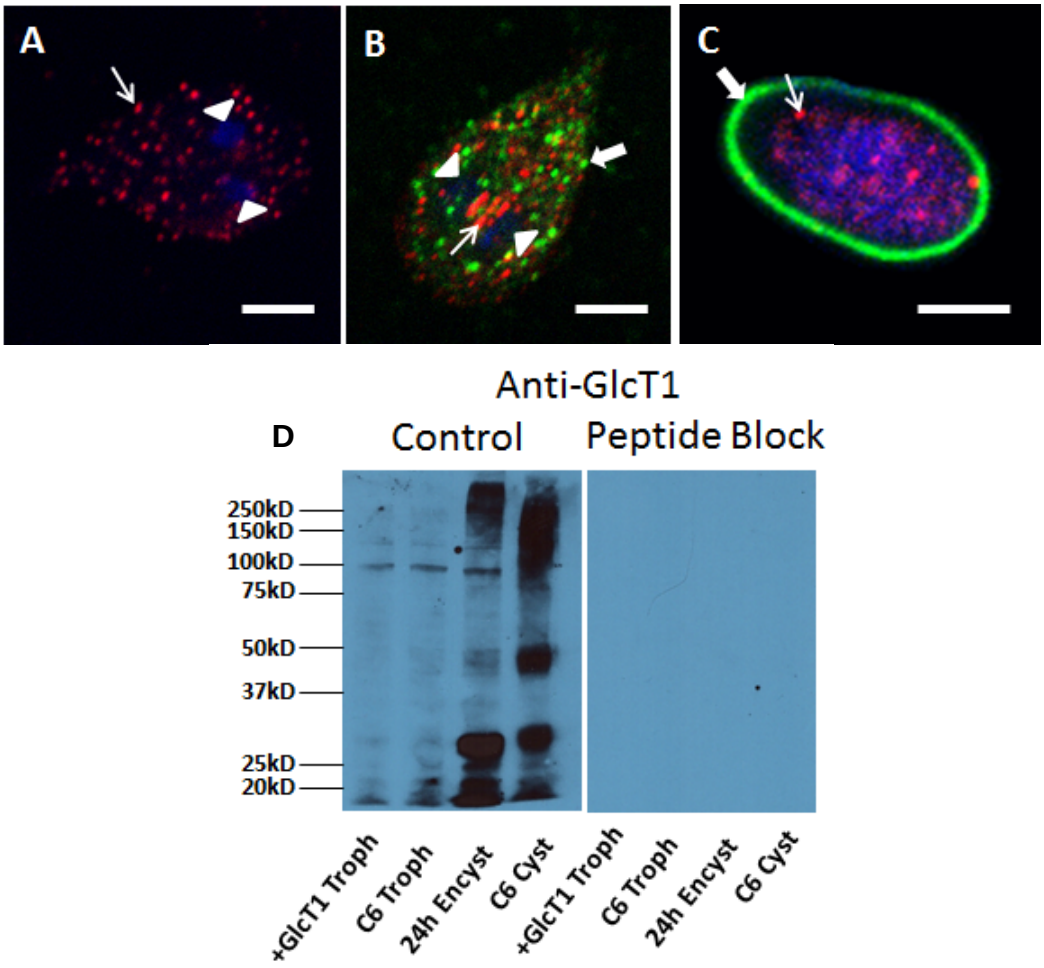

Figure 5: Immunofluorescence and western blot analysis of gGlcT1 custom antibodies

Giardial trophozoites (panel A), 24h encysting cells (panel B), and complete cysts (panel C) were fixed, and treated with both GlcT1 and CWP antibodies. In trophozoites, there is no CWP labeling but GlcT1 forms vesicle-like aggregates that are cytoplasmic. In 24h encysting cells, CWP antibodies label the ESVs that transport cyst wall material to the plasma membrane. On the other hand, GlcT1 continues to label the vesicle-like structures, but they do not localize with the ESVs. Moreover, GlcT1 begins to localize between the nuclei in encysting cells where basal bodies and central mitosomes are located. When cyst wall is completely formed, GlcT1 is again cytoplasmic and shows no uniform localization pattern. Purified polyclonal rabbit antibodies were also observed on immunoblot (panel D) and showed that GlcT1 labels proteins approximately ~30 and ~50 kD and the labeling increases as encystation

progresses. When blocked with peptide, no proteins were detected showing the specificity of the antibody.

2.2.2 The biogenesis of encystation-specific vesicles is modulated by gGlcT1 activity:

Giardia has a relatively simple life cycle—i.e., replicative trophozoites and relatively dormant cysts. During encystation in the small intestine, trophozoites synthesize ESVs which are necessary to transport CWPs to plasma membranes (Bittencourt-Silvestre et al. 2010). ESVs are synthesized in the endoplasmic reticulum (ER) located in the perinuclear membranes/cytoplasm in early encysting cells, and then distributed extensively in the periphery of the cell (in close proximity to peripheral vacuoles) in the late encystation phase (McCaffery et al. 1994). Because of the presence of Golgi-like cisternae in ESVs, these sorting vesicles are also considered as a primitive Golgi complex of *Giardia* (Stefanic et al. 2009). Over the years, many investigators have studied encystation and identified various proteins and genes that are associated with the process (Lauwaet et al. 2007). However, nothing is known about how the ESV biosynthesis is regulated and participates in viable and infective cyst formation. Because gGlcT1 activity is upregulated during encystation (**Fig. 4*A***), it was thought that gGlcT1 was linked to ESV biogenesis. To test this, control and gGlcT1-knockdown trophozoites were subjected to encystation for 18 h and ESV synthesis was monitored using an anti-cyst antibody that recognizes early and late ESVs as well as the cyst walls (Hehl et al. 2004). It was observed that anti-gGlcT1 morpholino oligonucletide, which inhibited gGlcT1 activities in trophozoites and cysts was found to block ESV biogenesis completely (**Fig. 6B**). On the other hand, ESV synthesis by encysting cells that were transfected with scrambled morpholino oligonucleotide (labeled as control [scrambled] in **Fig. 6*A***) was not affected at all. To further elucidate the role of gGlcT1 in ESV biogenesis, we overexpressed the *gglct1* gene in trophozoites and subjected them to encystation. **Fig. 6D** shows that *gglct1* overexpression produces aggregated and enlarged ESVs as compared with control cells that were transfected with empty plasmid. This indicates that the modulation of gGlcT1 activity either by knockdown or overexpression affects ESV biosynthesis in *Giardia*.

Fig. 7*A* demonstrates that gGlcT1 activity in overexpressed trophozoites increased ~3 fold as *compared with the activity present in control trophozoites (i.e., specific activities increased from 0.5* pmol/min/mg protein in control to 1.4 pmol/min/mg protein in gGlcT1-overexpressing cells). Knockdown by anti-*gglct1* morpholino oligonucleotide inhibits gGlcT1 activity in trophozoites by ~50% (from 0.5 pmol/min/mg protein to 0.24 pmol/min/mg protein). Furthermore, the rescue

experiment (i.e., transfecting gGlcT1-overexpressing trophozoites with anti-*gglct1* morpholino oligonucleotide) lowered the gGlcT1 activity by ~40%. **Fig. 7*B*** demonstrates that overexpression of gGlcT1 in non-encysting trophozoites causes an increased synthesis of NBD-GlcCer, further suggesting that gGlcT1 activity is directly linked to GlcCer synthesis in *Giardia*.

Next, we asked whether gGlcT1 modulation by overexpression and knockdown also affects the GSL profiles in *Giardia*. For this, lipids were extracted from overexpressed, knockdown, and rescued trophozoites and subjected to ESI-MS/MS analysis as described in experimental procedures. Using total-ion map and precursor-ion scans we identified both neutral and acidic GSLs that were present in control and gGlcT1-modulated trophozoites. Among the neutral lipids, mono-, di-, and tri-hexosylceramides were identified and found to be altered by gGlcT1 modulation. While gGlcT1 overexpression increased the amount of mono-hexosylceramide by ~28 fold, the effect was somewhat less (~ 2 fold) on di-hexosylceramides. Interestingly, no effects of gGlcT1 overexpression were observed in the case of tri-hexosylceramides (**Fig. 7*C***). gGlcT1 knockdown, however, elevated the level of mono-hexosylceramides (~5 fold) and the rescue experiment had no effect, i.e., same as the knockdown (Fig. 3*C*). Knockdown also decreased the levels of di- and tri-hexosylceramide slightly and rescue treatment appeared to reverse these effects (**Fig. 7*C***). The analysis of acidic fractions revealed that GM1, GM3, and GD3 lipids are present in control, overexpressed, knockdown, and rescued trophozoites; however, the changes of their levels were not consistent with gGlcT1modulation (not shown).

Because ESV synthesis is linked to gGlcT1 activity (**Fig. 6**), and the fact that PPMP blocks encystation (Hernandez et al. 2008; Sonda et al. 2008), we asked if the modulation of gGlcT1 activity (shown in **Fig. 8**) also regulates ESV biogenesis. Photographs **Fig. 8*A, b*** and **Fig. 8*A, c*** demonstrate that while gGlcT1 overexpression induces the synthesis of aggregated and enlarged ESVs, knockdown of gGlcT1 activity by anti-*gglct1* morpholino oligonucleotide completely blocks ESV synthesis. More interestingly, the rescue experiment reverses the effects of overexpression and knockdown and generates ESVs (**Fig. 8*A, d***) that are comparable to control cells (**Fig. *8A, a***). Again, **Fig. 8*A, e*** represents ESVs generated by encysting cells that were treated with scrambled morpholino oligonucleotide, which shows the similar pattern of ESVs found in non-transfected control encysting cells shown in **Fig. 8*A a.***

Analysis of individual ESVs are shown in **Fig. 8*B***, demonstrating that gGlcT1 overexpression increases the perimeters and areas of ESVs by ~6- and ~10 fold, respectively. The perimeter and area analyses of individual ESVs were carried out with the help of Zeiss Zen 2009 software (Carl Zeiss) as shown in **Fig. 4*C***.

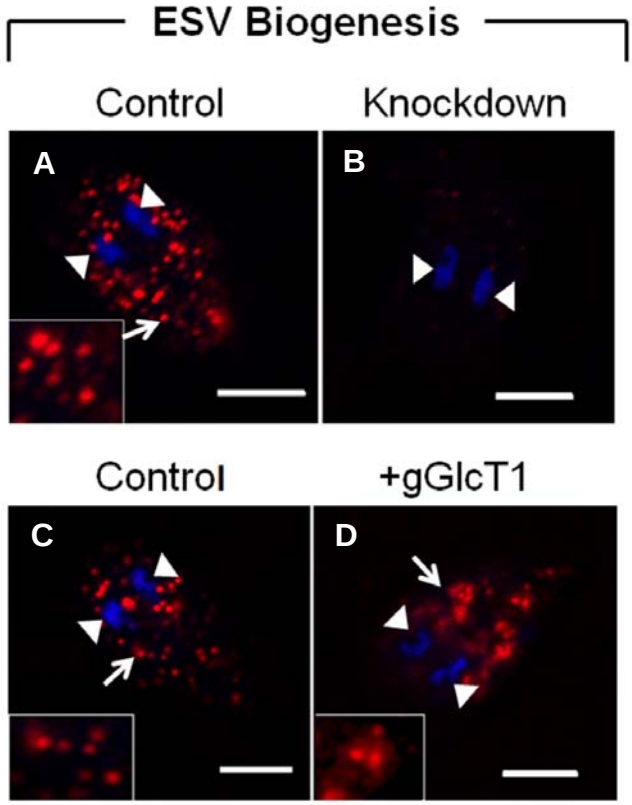

Figure 6: Knockdown and overexpression of gGlcT1 affects ESV biogenesis

Giardia trophozoites were transfected with anti-gGlcT1 morpholino oligonucleotide (50 M) (23) and subjected to encystation for 24 h before ESVs were analyzed by confocal microscopy. Control trophozoites (panel A) were transfected with a scrambled morpholino oligonucleotide sequence (50 M) supplied by the manufacturer. Discrete ESVs are visible in control cells but are not present in anti-gGlcT1 morpholino-transfected (i.e. gGlcT1 knockdown) cells (panel B). Arrows show ESVs, and arrowheads indicate nuclei. Bar, 10 m. B, gGlcT1- overexpressing (gGlcT1) trophozoites (panel D) were subjected to encystation for 24 h, and ESVs were analyzed as described above. gGlcT1 overexpression causes an aggregation/enlargement of the ESVs when compared with control cells that were transfected with empty plasmids (panel C). Arrows show ESVs, and arrowheads indicate nuclei. Bar, 10 m. Inset shows the magnified images of ESVs.

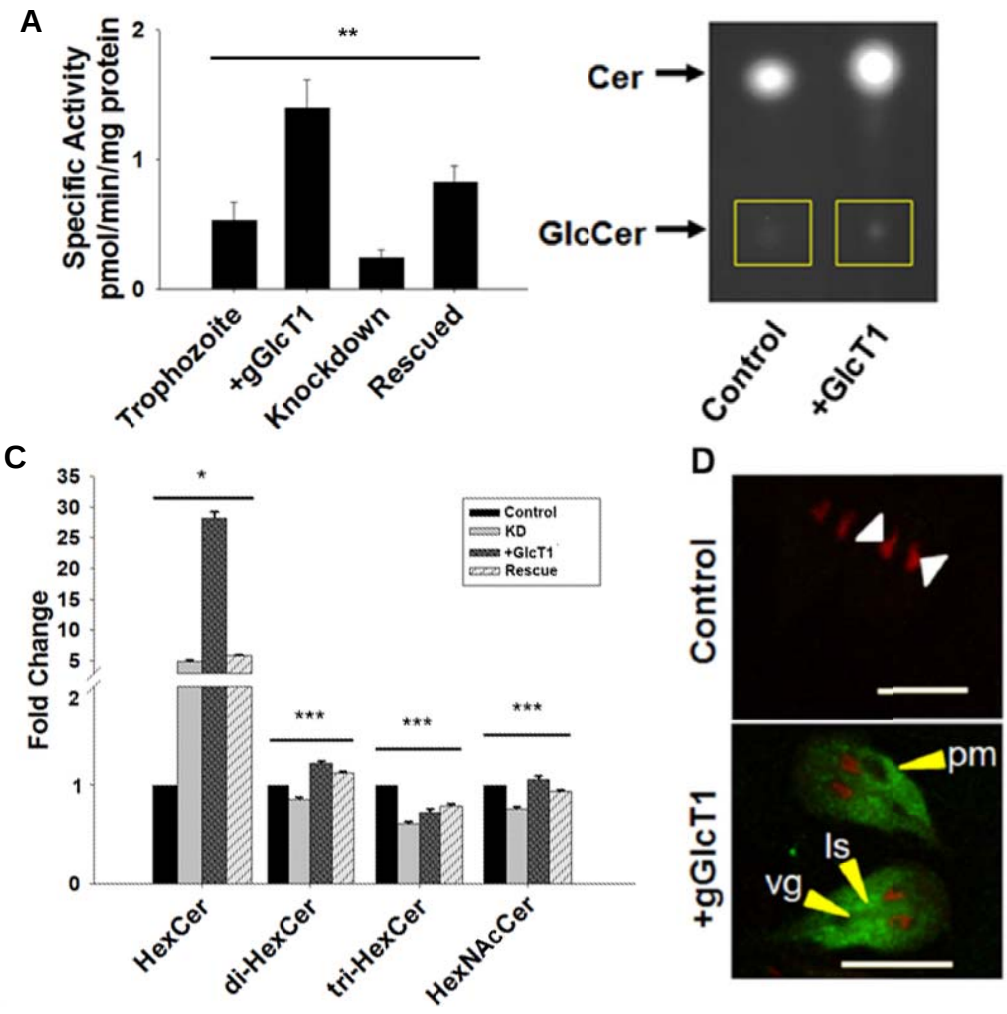

Figure 7: Modulation of gGlcT1 activity by overexpression and knockdown

A) stable *Giardia* cell lines overexpressing GlcT1 enzyme (designated as +GlcT1) were generated by transfecting trophozoites with pNT5-gglct1 (tagged with a small peptide, called AU1) plasmid. Overexpression increased the synthesis of gGlcT1 activity by 3-fold, which could be reduced by transfecting the overexpressed cells with anti-gGlcT1 morpholino oligonucleotide (i.e. gGlcT1-rescued cells). The enzyme activities in gGlcT1-overexpressing, -knockdown, and –rescued cells were measured and the results are presented in mean values S.D. of three separate experiments (**, $p<0.01$). B) synthesis of NBD-GlcCer by control and gGlcT1 overexpressed (+gGlcT1) trophozoites. Fractions containing NBD-GlcCer were extracted and spotted on a TLC plate and visualized under a UV lamp. It was noted that gGlcT1-overexpressing cells take up more ceramides than the control cells. Although the experiments were carried out twice separately with different cell preparations, results (TLC) shown here

are from a single study. C) ESI-MS/MS analysis of GSLs. GSLs from control (containing empty plasmid) with, gGlcT1-knockdown (KD), overexpressed (+GlcT1), and rescued trophozoites were extracted and analyzed. Mono-hexosylceramide (HexCer), di-hexosylceramide (di-HexCer); tri-hexosylceramide (tri-HexCer); the results (pmol/10^6 cells) presented here are the mean of fold changes (compared with control trophozoites) S.D. of three technical replicates (*, $p < 0.05$; ***, $p < 0.001$). D) trophozoites expressing AU1-tagged gGlcT1 were monitored by reacting with anti-AU1 antibody followed by labeling with FITC-conjugated anti-mouse antibody and examination under an immunofluorescence confocal microscope. The figure shows that overexpressed gGlcT1-AU1 (shown as+gGlcT1 in the figure) forms structures that are granular, aggregated, and localized throughout the cytoplasm and, to some extent, in the perinuclear regions. However, the overexpressed gGlcT1 granules are also localized in the ventral groove and lateral shield areas of the trophozoites. White arrowheads indicate DAPI-stained nuclei (control) and long yellow arrowheads denote ventral groove (vg), lateral shield (ls), and plasma membrane (pm) in overexpressed trophozoites. Bar, 10 μm.

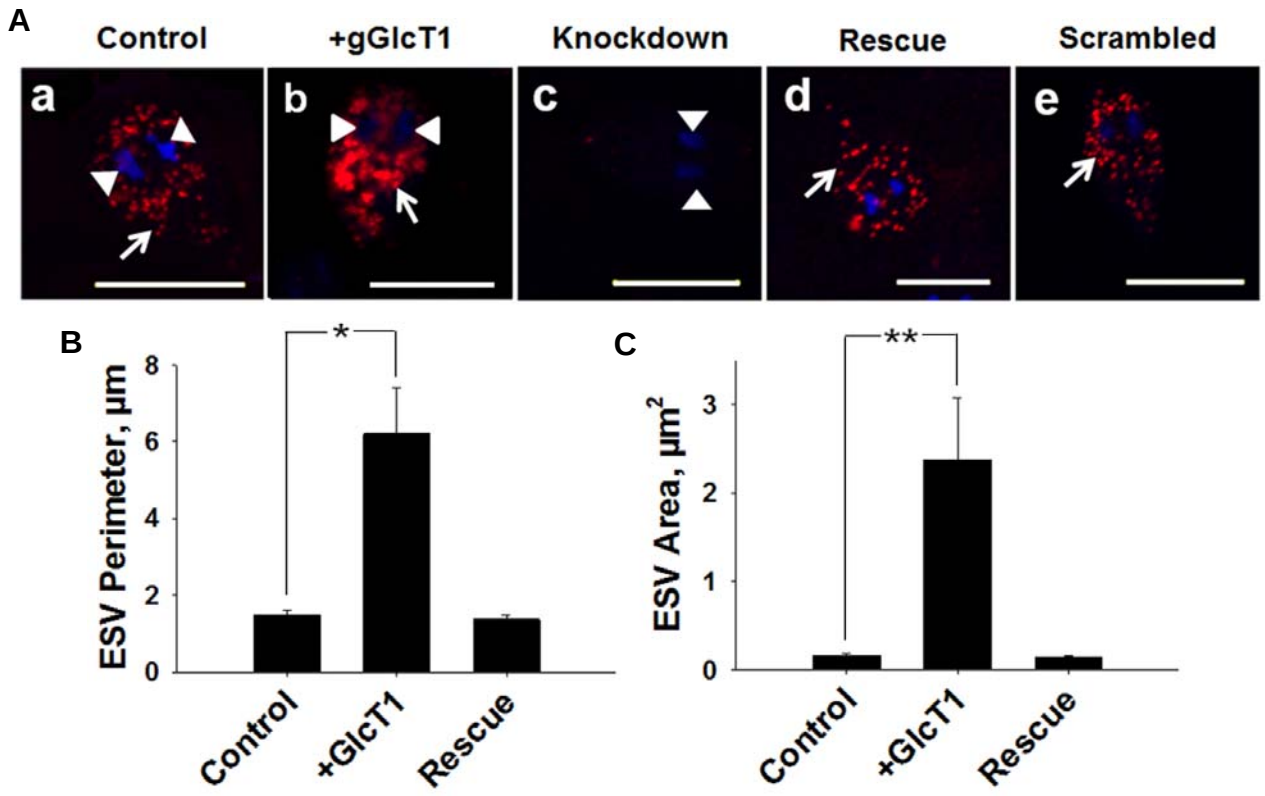

Figure 8: Modulation of gGlcT1 activity affects ESV biogenesis in *Giardia*

A, ESV production was monitored in the following:(panel a, control; panel b,GlcT1-overexpressing (+gGlcT1); panel c, antisense, morpholino oligonucleotide-treated gGlcT1-knockdown cells; panel d, rescued cells, and panel e, cells transfected with scrambled morpholino oligonucleotides. Arrows indicate ESVs, and arrowheads denote nuclei. Bar, 10m. B and C, changes of perimeters and areas of ESVs are shown in the bar graphs. gGlcT1 overexpressed encysting cells formed ESVs with a significantly larger area and perimeter. This morphology of ESVs reverted back to normal when gGlcT1-overexpressed cells were resxued with anti-gGlcT1 morpholinos. The analyses were carried out by measuring the perimeters and the areas of individual ESVs using Zeiss Zen 2009 confocal software. *, $p < 0.05$; **, $p < 0.01$.

2.2.3. Unlike PPMP, anti-gGlcT1 morpholino oligonucleotide does not affect cell replication of *Giardia* trophozoites:

PPMP is a common inhibitor of gGlcT1 enzyme that has been extensively used to evaluate GlcCer functions in various organisms (Kovacs et al. 2000). It has been reported that PPMP blocks replication and cytokinesis of giardial trophozoite and thereby thought to inhibit the encystation and cyst production in culture (Sonda et al. 2008; Stefanic et al. 2010). We have shown earlier that PPMP interferes with the formation of cysts when it is added in the culture medium during encystation (Hernandez et al. 2008). Because our current results show that anti-*gglct1* morpholino oligonucleotide inhibits gGlcT1 activity and ESV biogenesis (**Figs. 4 and 6**), and because the activity of this enzyme is low in trophozoites and upregulated in encysting cells, we thought that the effect of PPMP-inhibiting replication and cytokinesis on non-encysting trophozoites might not occur via GlcCer synthesis, as previously thought (Sonda et al. 2008; Stefanic et al. 2010). In fact, there are reports in which PDMP (an analogue of PPMP) has been shown to block cell-cycle progression in mammalian cells by inhibiting cyclin-dependent kinases, which could be independent of gGlcT1 inhibition and ceramide accumulation (Rani et al. 1995). To address this possibility in *Giardia*, trophozoites were transfected with morpholino oligonucleotide (anti-*glct1*) and the growth was measured as shown in **Fig. 9*A***. Side by side, the growth of the trophozoites in the presence of PPMP (10 μM) was also conducted, and the results showed that while PPMP affects the growth of trophozoites, anti-*gglct1* morpholino exhibits no effects (**Fig. 9*A***). **Fig. 9*B, b*** demonstrates that PPMP, as shown earlier (Sonda et al. 2008), blocks the replication of trophozoites and generates undivided-doublet cells. In contrast, anti-*gglct1* morpholino did not affect the replication of trophozoites (**Fig. 9*B, c***). When morpholino-transfected, gGlcT1-knockdown trophozoites were treated with PPMP, the formation of undivided doublets was observed again (**Fig. 9B, d**). The analysis revealed that while ~25% of trophozoites form undivided doublets after PPMP treatment, less than ~5% of doublets were observed in control and anti-gglct1 morpholino-treated trophozoites (**Fig. 9C**). The combined effect of anti-gglct1 morpholino and PPMP generated ~20% doublets, as shown in **Fig. 9C**. These results indicate that PPMP may not act via gGlcT1 in *Giardia* and that the blocking of cytokinesis in trophozoites by this inhibitor could be through other mechanisms.

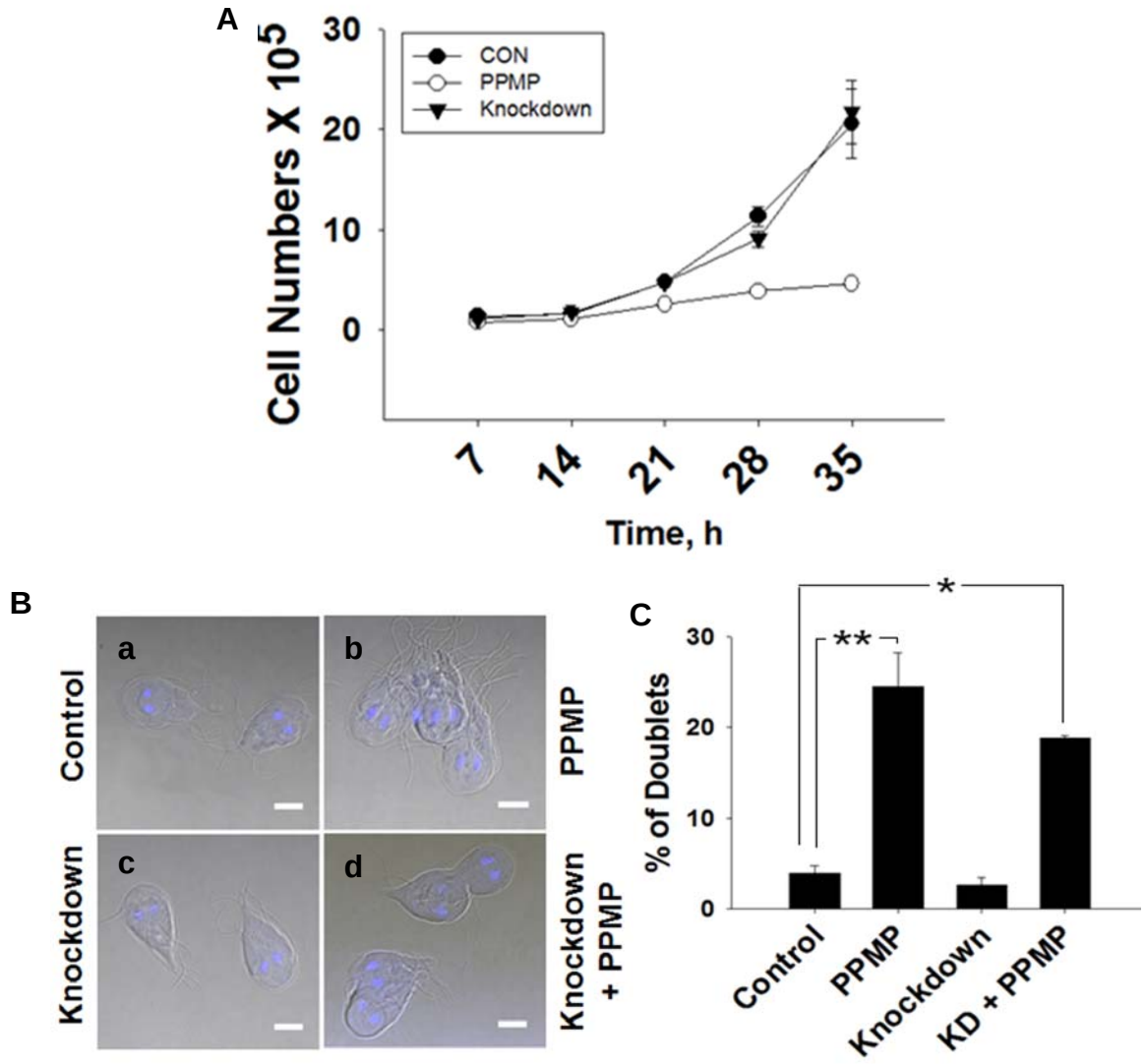

Figure 9: Effects of PPMP and anti-gGlcT1 morpholino on growth and replication of trophozoites

A) Assessing the growth of trophozoites. Approximately $2X10^5$ cells were inoculated into 4-ml tubes containing TYI-S-33 medium, pH 7.1. Trophozoites were allowed to grow for 35 h (the doubling time of trophozoites is ~7 h) and were counted under a microscope. Control and Morpholino-treated cells maintained a normal growth rate whereas the cells treated with PPMP had slow growth. Each experiment was carried out in triplicate, and the experiment was repeated three times. B) Differential interference contrast confocal images show the effects of anti-gGlcT1 morpholino oligonucleotide and PPMP on cell division and cytokinesis. Nuclei were stained with DAPI. Control, panel a; 10µM PPMP, panel b; knockdown with morpholino, panel c; morpholino + PPMP cells, panel d. C) Quantitative

assessment (e.g. microscopic counts) of doublet trophozoites that are produced by control, PPMP-treated, anti-gGlcT1 morpholino-induced knockdown trophozoites (knockdown) and knockdown (KD) trophozoites + PPMP. At least 100 singlet, doublet, and triplet (the number of undivided triplet trophozoites were small and therefore are not shown in the figure) trophozoites from 10 different fields were viewed and counted under the Zeiss LSM 700 confocal microscope using Zen 2009 software. The percentage values of doublet cells were calculated based on the total number of singlet, doublet, and triplet cells. *, $p < 0.05$; **, $p < 0.01$.

2.2.4 Regulated expression of gGlcT1 is critical for maintain the cyst viability:

The production of viable cysts is essential for *Giardia* to establish infection in the small intestine of humans. Earlier reports suggested that cyst morphology is directly correlated with cyst viability and only viable cysts of *Giardia* produce infection in mice (Schupp et al. 1987; Schupp et al. 1987). Gillin et al. (1989) reported that in vitro-derived *G. lamblia* cysts are of two kinds—i.e., Type I and Type II. The Type I cysts are oval-shaped with uniform and refractive cyst walls, being mostly (~90%) viable. In contrast, Type II cysts lack the morphological characteristics of Type I and a majority (~70%) of them are non-viable. In a heterogeneous cyst population (i.e., a population composed of both Type I and Type II cysts), ~10-30% of cysts exhibit Type I morphology (Gillin et al. 1989; Boucher et al. 1990). Because we found that the gGlcT1 activity increased during encystation (**Fig. 4**) and that the modulation of its function (by overexpression and knockdown) regulated ESV biogenesis (**Figs. 4 and 6**), we asked whether gGlcT1 activity is also important for maintaining the cyst morphology and viability. Therefore, trophozoites from various conditions—i.e., control, and gGlcT1-overexpressing, -knockdown, and -rescued—were subjected to encystation for 72 h, and the cysts were isolated by centrifugation as described in the experimental section. **Fig. 10*A***, ***a*** shows control cysts with Type I morphology that react with the anti-cyst antibody, which labels the oval-shaped cyst wall. However, this changes significantly in gGlcT1-overexpressing and -knockdown cells (**Figs. 10*A*, *b*, and 10*A*, *c***). Cysts produced by gGlcT1-overexpressing cells appear to be Type II because they are incomplete, clustered, have thin cyst walls, and show minimum reactivity to anti-cyst antibody. Knockdown of gGlcT1 also produces aggregated cryptic cysts; the majority of these cyst-like structures exhibit no reaction to anti-cyst-wall antibody. The rescue treatment, shown in **Fig. 10*A*, *d***, however, produces cysts with well-formed cyst walls that react with the cyst antibody. Because the morphology could be an indicative of viability (Schupp et al. 1987; Boucher et al. 1990), our next goal was to test if the viability of cysts is affected by gGlcT1-overexpression and -knockdown. The viable and non-viable cysts were identified by staining with fluorogenic dyes—i.e., cell permeable esterase-substrate fluorescein diacetate (FDA) and the cell impermeant nucleic-acid stain propidium iodide (PI), which were earlier used by other laboratories to determine the viability of giardial cysts (Schupp et al. 1987; Schupp et al. 1987; Gillin et

al. 1989). **Fig. 10*B*** indicates that while in control samples ~12% of cysts were viable, only ~8% and ~3% of cysts were viable in gGlcT1-overexpressing and -knockdown cells, respectively. Most importantly, the rescue experiment, in which the effect of gGlcT1 was neutralized by anti-gGlcT1 morpholino analogue, recovers the cyst and increases the viability up to ~10%. This is an important observation and strongly suggests that gGlcT1 activity in *Giardia* not only regulates ESV biogenesis but also maintains the cyst viability. Although it appears that these numbers of viable cysts are somewhat low, our calculation is based on the total number of water-resistant cysts that contain all type of cysts, including Type I and Type II. Thus, viability estimations, shown here, are within the expected range and in accordance with the report published by Gillin et al. (1989) and Boucher et al. (1990).

2.3 CONCLUSION

Glycospingholipids (GSLs) are known to participate in various cellular functions, including differentiation, signaling, and apoptosis. GlcCer, an important GSL, is involved in inducing the drug-resistance phenomenon in cancer cells, as well as determining fungal pathogenesis and the formation of Polycystic Kidney Disease in humans (ref). In *Giardia*, I found that GlcCer plays an important role in generating viable cysts. In Chapter 1, I showed for the first time that the activity of gGlcT1 (which catalyzes the synthesis of GlcCer) increases during encystation. PPMP has been found to inhibit gGlcT1 activity in trophozoites and early encysting cells, but it is not effective in reducing the activity of the cyst gGlcT1. When gGlcT1 activity was modulated by using the overexpression and knockdown of the *gglct1* gene, I found that ESV biogenesis is affected. Contrary to previous reports (Sonda et al. 2008; Steffanic et al. 2010), I have demonstrated that PPMP is active during encystation but not in the non-encysting stages of the parasitic life cycle. Most importantly, it was observed that the regulated expression of gGlcT1 is important for maintaining cyst viability. This study will set the stage for future investigations to develop potential therapies targeting GlcCer biosynthesis in *Giardia*. In recent years, efforts have been underway to produce a vaccine against *Giardia* trophozoites (Rivero et al. 2010), and so my results should provide valuable information about whether gGlcT1 knockdown-attenuated cysts with reduced viability could be used as a live vaccine candidate against giardiasis in the future.

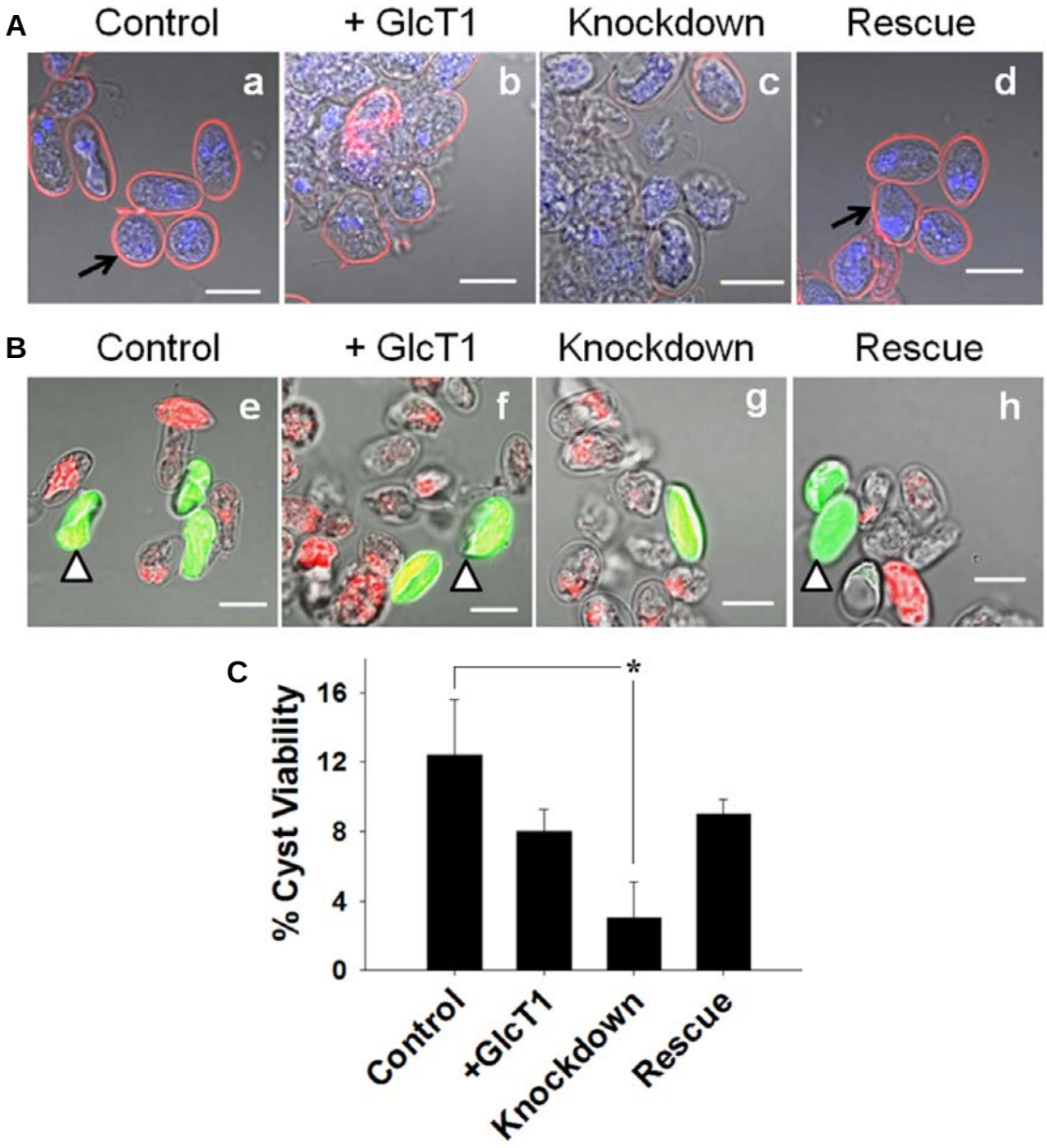

Figure 10: gGlcT1 activity regulates the morphology and viability of in vitro-derived cysts

A) trophozoites were subjected to encystation for 72 h. Cysts were harvested and treated with anti-cyst antibody to be examined under differential interference contrast and confocal microscopy. Panel a, control. Panel b, gGlcT1-overexpressing (+gGlcT1); panel c, gGlcT1-knockdown; and panel d, rescued. Arrows indicate the cyst wall. Cyst nuclei were stained with DAPI. gGlcT1-overexpressed and knockdown cells form broken and incomplete cysts compared to control, meanwhile rescue cysts form control-like cysts. Bar, 10 µm. B) Testing the same cyst samples (shown in A) for viability using FDA

and PI stains. The cells were also analyzed using differential interference contrast and confocal microscopy with green indicating live and red denoting dead cysts. Panel e, control; panel f, +gGlcT1; panel g, gGlcT1 knockdown, and panel h, rescued. Bar, 10 μm. C) Quantification of the viability assay. Approximately 100–150 cysts were examined from 5 to 10 different fields to identify and count the viable cysts. Viability in +GlcT1 and knockdown cysts are lower than control. However, the viability of rescue cysts increases slightly to control levels. The results were presented in mean values ±S.D. of three separate experiments conducted in different days with different sample preparations. *, $p < 0.05$.

Chapter 3: Glucosylceramide Transferase regulates ESV biogenesis and cyst viability by maintaining the lipid homeostasis in *Giardia*

The encystation-specific vesicles (ESVs) are considered post-ER or transient Golgi organelles that transport all cyst-wall materials to the plasma membrane of trophozoites. Usually, these vesicles are round and enclosed by bilayered membranes (Lanfredi-Rangel et al. 2003). When they reach the plasma membranes carrying CWPs, these vesicles fuse with the membranes and release all their contents to form solid and protective cyst walls. Thus, proper integration of ESVs to plasma membranes is critical for cyst-wall biogenesis and the production of viable and infective cysts. Because my results in Chapter 2 show that the modulation of gGlcT1 activity (by overexpression, knockdown, and rescue) affects ESV biogenesis and cyst viability, and because gGlcT1 overexpression produces enlarged ESVs and non-viable cysts, I thought that gGlcT1 could be linked to the uptake and maintenance of the overall of balance of lipids in *Giardia* and that changing this balance might produce abnormal ESVs and non-infective cysts.

As far as giardial lipid metabolism is concerned, earlier studies have indicated that this parasite has limited lipid synthesis ability and that the majority of its lipids and fatty acids are obtained from the culture medium, of which the two major ingredients are bovine serum and bile. The serum and bile provide essential lipids to *Giardia*, including phospholipids, cholesterol, fatty acids, and sphingolipids. We have also reported earlier that although *Giardia* is dependent completely on exogenous lipids it has the ability to generate selective membrane lipids that are not present in the medium (reviewed by Yichoy et al. 2011). Studies from our laboratory indicate that phospatidylcoline (PC) and phosphatidylglycerol (PG) are the major phospholipids in *Giardia*. Mass spectrometric analysis revealed that at least 17 and 19 different species of PGs and PCs, respectively, are present in this parasite, along with various combinations of odd- and even-numbered, carbon-containing fatty acids. In addition to the PGs and PCs, 6 species of phosphatidylethanolamines (PEs), 3 species of sphingomyelin (SMs), and 2 species of phosphatidylinositols (PIs) were also present. It is interesting that except for PGs and PEs all phospholipids are obtained from the growth medium, which means that it is likely that PGs and PEs are

newly synthesized phospholipids in *Giardia* (Yichoy et al. 2009). Sphingolipidomic analysis indicated that sphingomyelin; mono-, di- and, tri-hexosylceramides; and gangliosides (GM1, GM3, and GD3) are the major GSLs found in this organism. These GSLs display profiles similar to those present in the serum (except for some variations that we found in fatty-acid chain lengths; Duarte et al., manuscript in preparation).

Therefore, the lipid metabolism in *Giardia* is a complex scenario in which the parasite takes up the majority of its lipids from the medium and synthesizes or assembles a few. It is not well understood how these two separate events (i.e., uptake and de novo synthesis/assembly) are coordinated and utilized for ESV biogenesis and cyst production. To answer some of these questions, and to understand how lipid metabolism is regulated by gGlcT1, I overexpressed gglct1 gene and investigated lipid internalization and membrane fluidity by *Giardia* trophozoites.

3.1 MATERIALS AND METHODS

3.1.1 Determination of mRNA levels of giardial lipid genes by quantitative reverse transcription-PCR (qRT-PCR)

To determine whether the mRNA expression of various lipid genes are altered in gGlcT1 overexpressed *Giardia*, RNA from encysting and non-encysting trophozoites as well as from water-resistant cysts were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized by using 2µg of purified RNA with the iscript cDNA synthesis kit (BioRad). 2µl of a 1:20 dilution of cDNA was mixed with 10µl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), the primers for the genes of interest (1µl each) and run through the MyiQ Thermal Cycler using the following parameters: 95°C 15min, 55 X (95°C: 30 sec, 55°C: 30 sec, 72°C: 30 sec), 72°C: 5 min and 60°C: 30 sec for melt curve. Tubulin was used as a housekeeping gene the relative standard curve method was used to quantify transcript levels. For the list of primers that were analyzed are shown in Table-1.

3.1.2 Sterol analysis by GC-MS

Sterol analysis in control and gGlcT1-overexpressing trophozoites was carried out according to the method of Fridberg et al. (2008). Trophozoites (~1x108) were grown and harvested as described

above; resuspended in CHCl3: MeOH: H2O (1:2:0.8, v/v/v), and centrifuged (1500 x g) at room temperature for 15 min. The supernatant was collected and dried under nitrogen, and the pellet was then extracted with CHCl3: MeOH (2:1, v/v) three times and with CHCl3: MeOH: H2O (1:2:0.8, v/v/v) twice before being dried under a nitrogen stream. The supernatants obtained from these six extractions were pooled together before being dried in the same flask. The dried samples were then submitted to Folch's partition, being first diluted in CHCl3: MeOH: H2O (4:2:1.5, v/v/v), and then centrifuged (1500 x g) at room temperature for 15 min. This partitioning gave rise to two phases: a lower phase, rich in free fatty acids, sterols, and phospholipids, and an upper phase, rich in inositolphospholipids. The lower phase was dried down with nitrogen gas, resuspended in pure CHCl3, and fractionated through a silica-gel 60 column according to Pernet et al. (2006). The CHCl3 phase of the samples were dried under a nitrogen stream and dissolved in dichloromethane. Fifty ng of external standard (Stigmasterol, Sigma Aldrich) was added to the giardial sample and 1 µl of the resulting mixture was injected into the gas chromatographer (Trace GC, Thermo Fisher Scientific) coupled to a mass spectrometer (Polaris Q, Thermo Fisher Scientific) (GC-MS). The fractionation was performed in a TR-5-ms column (30 m x 250 mm x 0.25 µm, Thermo Fisher Scientific). The injector temperature was set at 250 °C. The gradient was set at an initial temperature of 170 °C for 3 min, followed by a 20 °C /min increase up to 280 °C, and then a 17-min hold after the final temperature was achieved. The carrier gas was helium, with a flow rate of 1.2 ml/min. The samples were ionized by electron impact (EI) at 70 eV and 30 °C, and the spectra were collected at the 50–650 m/z range.

3.1.3 Fatty acid methylation and GC-MS analysis

Fatty acids extracted from *Giardia* were subjected to methylation following the protocol described by Maldonado et al. (2006). Briefly, a methanol fraction of lipid extracts from trophozoites (~1x108) was dried under nitrogen and resuspended in 13 (N) NH4OH: MeOH (1:1, v/v) and incubated for 1 h at 37 °C. The sample was then dried under a nitrogen stream, resuspended in MeOH, dried again, and mixed with 0.5 (N) methanolic HCl. The mixture was incubated for 1 h at 75 °C and then mixed with 0.5 (N) NaOH to neutralize the reaction before mixing with 750 □l dichloromethane and H2O (1:1, v/v). This mixture was then centrifuged at 1500 x g for 15 min and the lower phase was separated.

Standards were dissolved in dichloromethane and analyzed by GC-MS (Trace GC/Polaris Q, Thermo Fisher Scientific). The injector was set at 200 °C, and the following gradient was used: 70 °C for 5 min, followed by 4 °C/min up to 140 °C, 2 °C/min up to 185 °C, and 185 °C for 10 min. Helium was used as the carrier gas, with a flow rate of 1 ml/min, then the molecules were ionized by electron impact at 70 eV and 200 °C. The spectra were collected at the 30−400 m/z range and fatty acid (FA) species were identified by comparison with the FAME 37 methylated FA mix standard (Supelco, Sigma Aldrich).

3.1.4 Membrane fluidity assay

Approximately 107 trophozoites (control and gGlcT1-overexpressing) were grown and harvested as described above, and the cells were analyzed using the Membrane Fluidity Kit (M 0271, Marker Gene Technologies, Inc., Eugene, OR). Cold-shock trophozoites (4 °C, 30 min) were used as positive control. The cells were resuspended in 200 µl perfusion buffer with 20 µM of fluorescent lipid reagent (pyrenedecanoic acid) and 0.08% of pluronic F127, provided with the kit. The suspension was shaken on a rocker for 1 h at RT, and then the cells were washed with PBS and subsequently analyzed using the ISS K2 Multifrequency Phase and Modulation Fluorometer (ISS, Inc., Champaign, IL). The samples were excited at 341 nm, and the emission spectra were recorded at 360–500 nm. Crossed polarizers on excitation and emission beams were used to reduce the background because of scatter. The emission spectra were then appended with normalized intensities using VINCI software (ISS).

3.1.5 Transmission Electron Microscopy

Cells were grown and encysted as described above. Attached parasites were placed in 2% paraformaldehyde/2.5% gluteraldehyde in PBS and left for 1 hour at room temperature. The cells were harvested, then washed with PBS then subsequently embedded and pelleted in 2% agarose. The sample was post-fixed in 3mL of 1% osmium tetroxide for 1 hour at RT. The cells were then washed with dH2O before en bloc staining with 1% uranyl acetate for 1 hour at RT.

The cells were then incubated in 1% uranyl acetate for 1 hour at room temperature. Afterwards, samples were washed with dH20 dehydrated in an ethanol series of 30%, 50%, 70%, 90%, and 100% at RT. Treatment with Propolyne Oxide (PO) was undertaken before cells were infiltrated with a Eponate 12 resin (EPON). Ninety nanometer (90 nm) sections of the sample was cut using a Leica Ultracut

UCT7 ultramicrotome before staining with 2% uranyl acetate and lead citrate. Sections were views under a JEOL 1200 EX transmission electron microscope equipped with an AMT 8 megapixel digital camera.

3.1.6 Statistical analysis

All values were given as mean values ± S.D., and statistical analyses were generated using SAS software ver. 9.2 (SAS Institute, Cary, NC) and Graphpad QuickCalcs (http://www.graphpad.com/quickcalcs/). Student's t-test was performed on fatty acid and sterol data. Statistical values less than 0.01 were considered highly significant, whereas values less than 0.05 were considered significant. The variances of MS data for GSLs were calculated by one-way ANOVA.

Table 1: Giardial Lipid genes analyzed by Real Time qRT-PCR

Giardial Lipid Genes	Accession No.	Primers
PI Synthase/CDP-DAG-inositol 3-phosphatidyltransferase (gPIS)	XP_001707169	For: TGGCAGTTTTCTGCTTTGTG Rev: AATCCGATCGCATACTTTGG
PGP synthase/CDP-DAG-glycerol-3-phosphate-3-phosphatidyltransferase (gPGPS)	XP_001707005	For: ATGTGGGTTCCTTCAGTTGC Rev: GAGACACGCTGTGATGGAGA
PS decarboxylase (gPSD)	XP_001707910	For: CTCCACTGGGCCGTATTTTA Rev: ATGAGCCTGACAGGATGACC
PS synthase (gPSS)	XP_001707737	For: CAGCCTTTGCCTTGCTTATC Rev: GCTAAGGTTGTGTGGCCATT
Phospholipid-transporting ATPase IA, putative (gPLTATPase 1A)	XP_001704967	For: ATGCACAATCGCATCCAGTA Rev: CAGTATCAGCGCCTTTGACA
Lysophosphatidic acid acyltransferase, putative (gLPAAT)	XP_001707002	For: GTCTCCCATCGCCGTAGATA Rev: CGAGTCCAAAACCAGAGAGC
Fatty acid elongase 1 (gFAELO)	XP_001708101	For: TGGCTTCAAATGCAACTCTG Rev: TCGCGTTCTAGACGATCCTT
Ceramide glucosyltransferase (gGlcT1)	XP_001704299	For: GCCAACCAGCTCATAATGGT Rev: ATTGACCTTCCCTGCTGATG
Acid sphingomyelinase-like phosphodiesterase 3b (gASMase)	XP_001709364	For: AGAGATGACCCAGCGAGCTA Rev: TACCCCCGTCTTTGAAACTG
Serine palmitoyltransferase-1 (gSPT1)	XP_001707207	For: TGATAGACTGCGCAACAACC Rev: CAGCTGCACTGGGATACAGA
Serine palmitoyltransferase-2 (gSPT2)	XP_001704960	For: GTGGGTTGAAGGAGATTGGA Rev: CCGTGTGTCCTGATGAAATG
Phosphoinositide-3-kinase, class 3 (gPI3K)	XP_001708644	For: TACTTCAACGCCATGCTCTG Rev: AAAGGCGTAGGGTGATTGTG

3.2 RESULTS

3.2.1 Lipid homeostasis is disrupted upon gGlcT1 overexpression in trophozoites

Because gGlcT1 overexpression induces the synthesis of enlarged vesicles and produces cysts with reduced viability (**Figs. 4, 6, 10**), we investigated whether gGlcT1 expression is associated with increased lipid uptake by *Giardia* trophozoites, I asked intracellular levels of cholesterol and fatty acid are altered by gGlcT1, which in turn would affect the membrane fluidity and lipid uptake.

gGlcT1 overexpression elevates the intracellular levels of cholesterol and fatty acid in trophozoites. Like other lipid molecules, *Giardia* also has a limited ability to synthesize cholesterol and fatty acids de novo, which are obtained from the growth medium. Interestingly, both cholesterol and fatty acids have been shown to be involved in regulating encystation and cyst formation by this waterborne pathogen (Lujan et al. 1996; Lujan et al. 1996; Yichoy et al. 2009). As gGlcT1 overexpression increased the internalization of NBD-ceramide from the medium (**Fig. 4**), we thought it would be important to examine whether gGlcT1 expression also changes the cellular fatty acid and cholesterol levels. Cholesterol and free fatty acids in control and gGlcT1-overexpressing cells were analyzed by GC-MS. The sterol analysis showed that cholesterol is the major sterol in both control and gGlcT1-overexpressing cells (**Fig. 11A**) and no other sterols, including cholesterylesters or ergosterol, were detected (Ellis et al. 1996). It is interesting that gGlcT1 overexpression increased the intracellular level of cholesterol by ~2 fold. Fatty acid analysis (**Fig. 11B**) revealed that palmitic acid, stearic acid, oleic acid, and linoleic acid were the major fatty acids present in *Giardia* trophozoites and that gGlcT1 overexpression increased the level of these fatty acids by ~20, ~22, ~25, and ~38%, respectively. Thus, as with other lipids as shown in Fig. 7, gGlcT1 expression also increased the uptake of cholesterol and fatty acids by *Giardia* (**Figs 11A and 11B**)

Because the changing of membrane fluidity may increase lipid uptake by trophozoites, we investigated the possibility that the membrane fluidity could be modified due to the upregulation of cholesterol and fatty acids by gGlcT1, allowing the cells to internalize excess NBD-ceramide as shown in **Fig. 4**. The membrane fluidity was measured using a fluorescent lipid probe, pyrenedecanoic acid, as detailed in the experimental procedure. This pyrene probe usually forms aggregates called excimers by

interacting with membrane components that causes a shift of emission spectrum to a longer wavelength. Therefore, the ratio of monomer to excimer shift could be utilized to measure the change of membrane fluidity (described by the manufacturer, Marker Gene Technologies). **Fig. 11C** shows that gGlcT1 overexpression did not produce pyrene excimers from monomers. On the other hand, the trophozoites treated at 4 oC (cold-shock) showed significant changes from monomer to excimer of the pyrene probe (**Fig. 11D**). This suggests that the fluidity of trophozoite plasma membranes is not affected by gGlcT1 overexpression although it increased the uptake of lipid, cholesterol, and fatty acids. We speculate that the increased lipid uptake by gGlcT1-overexpressed trophozoites occur via receptor- or raft-mediated lipid endocytosis (Lujan et al. 1996; Hernandez et al. 2007) rather than to the change of membrane fluidity.

3.2.2 gGlcT1 overexpression is associated with the alterations of lipid metabolic genes in *Giardia*

Lipid homeostasis plays a critical role in maintaining metabolic balance in eukaryotic cells (Hermansson et al. 2011). Recent results demonstrate that SLs play important role in maintaining the overall lipid homeostasis in mammalian cells and any changes of this balance may lead to various metabolic disorders (Bektas et al. 2010; Pewzner-Jung et al. 2010). A recent study in *Giardia* also indicates that the inhibition of GlcCer synthesis by PPMP increases palmitate incorporation and elevates the levels of diacylglycerol and other neutral lipids (Stefanic et al. 2010), implying a possible cross-talk between SL and other lipid metabolic pathways. We found that that gGlcT1 overexpression not only alters the intracellular labeling of lipids (**Fig. 4**) but also produces enlarged, aggregated ESVs. Based on these results, it can be hypothesized that gGlcT1-regulated lipid homeostasis is critical for ESV formation. Therefore, to gain an overall understanding of lipid gene expression in gGlcT1 overexpressed *Giardia*, we carried out qRT-PCR analyses. Briefly, control (transfected with empty plasmid) and gGlcT1 overexpressed trophozoites were subjected to encystation and eleven genes representing SLs and PLs metabolism (Table-1) were analyzed. Results (**Fig.12**) show that except

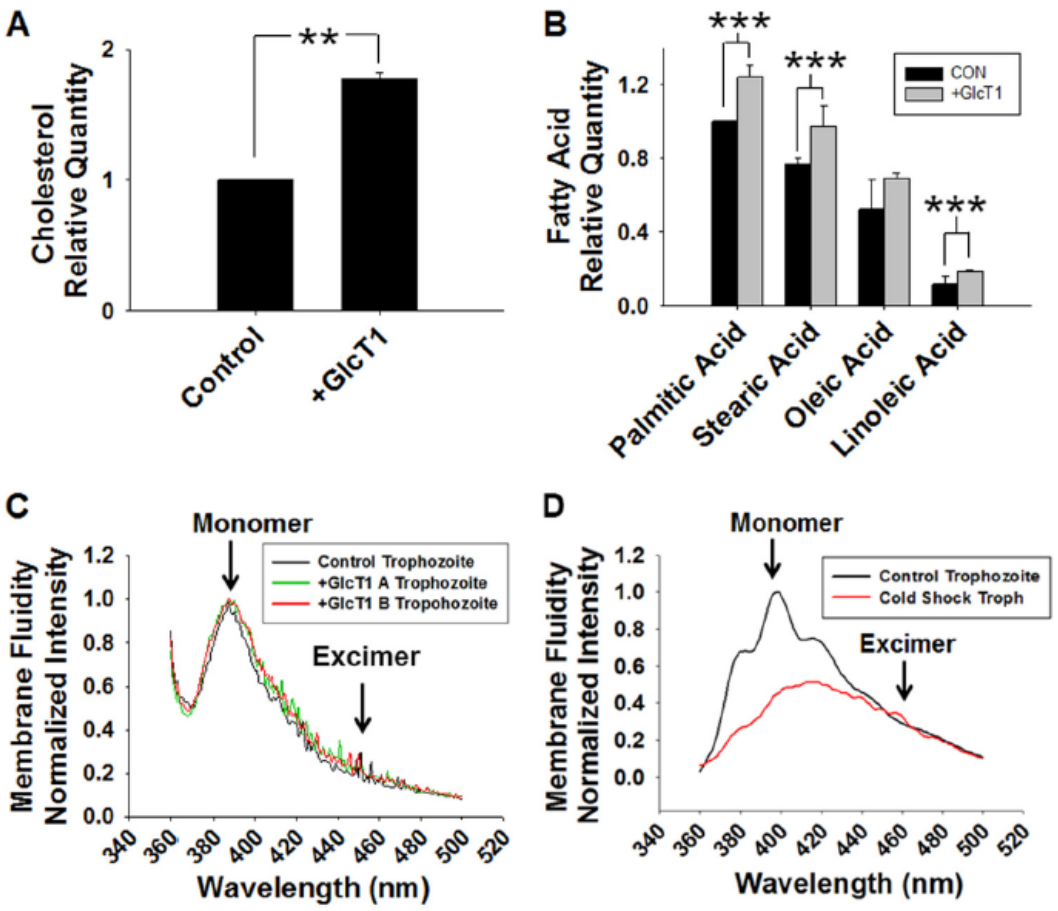

Figure 11: gGlcT1 overexpression increases cholesterol and fatty acid levels but does not alter membrane fluidity

Control and gGlcT1-overexpressing *Giardia* were subjected to Folch's partition and subsequent fractionation to separate sterols and fatty acids. A) Sterols were extracted in a solvent containing chloroform, methanol, and water followed by GC-MS analysis. Stigmasterol was added as an external standard to quantify cholesterol. When compared to control, the amount of cholesterol present in +GlcT1 is ~2-fold higher. The experiment was carried out in triplicate, and data are shown as relative quantity ±S.D. of two separate experiments (**, $p<0.01$). B) Fatty acids extracted from *Giardia* were subjected to methylation and analyzed by GC-MS. Similar to cholesterol results, the amount of fatty acids also increased in +GlcT1 trophozoites. The experiment was carried out in triplicate, and data are shown as relative quantity ±S.D. of two separate experiments (***, $p<0.001$). C) membrane fluidity was assessed using a fluorescent lipid, pyrenedecanoic acid. The monomer/excimer ratio was calculated by normalizing fluorescence intensities. Control (black line, transfected with empty plasmid) and gGlcT1-

overexpressed (gGlcT1) cells (red and green lines, two separate gGlcT1-overexpressing trophozoite samples) are shown and there no change in membrane fluidity between the cell types. D) changes of membrane fluidity in cold-shock trophozoites. The black line indicates control trophozoites incubated at 25 °C for 30 min, and the red line denotes cold-shock trophozoites kept at 4 °C also for 30 min before conducting the. In both cases (C and D) experiments were repeated several times with different cell preparations, and the results shown here came from a single experiment.

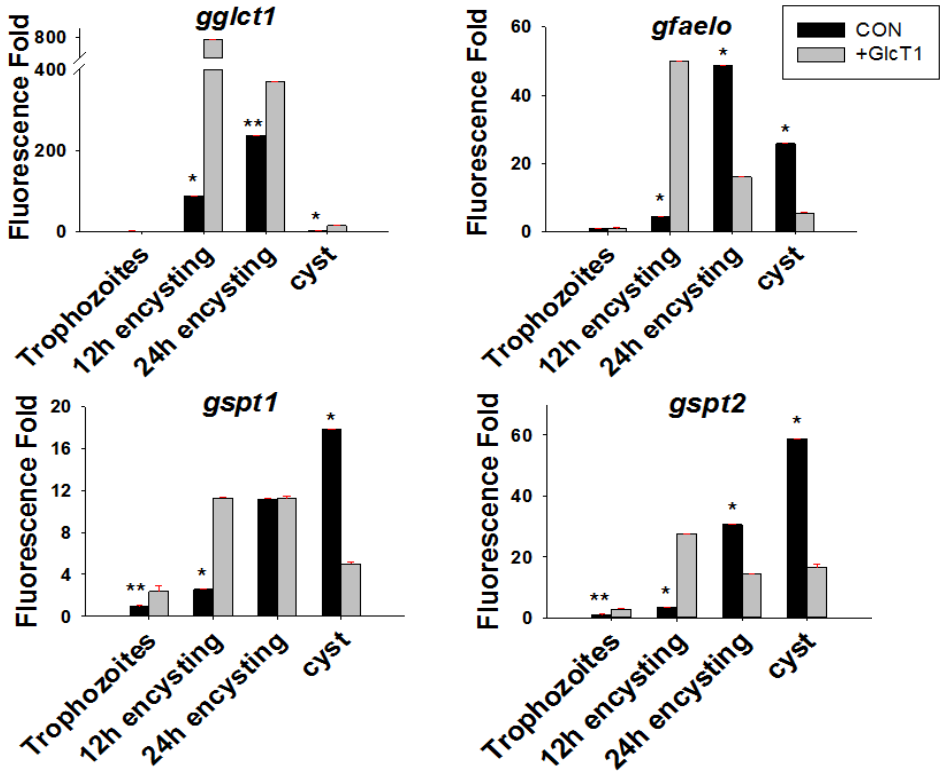

Figure 12: Real Time qRT-PCR Analysis of Lipid Genes in gGlcT1 Overexpressed *Giardia*

Real Time qRT-PCR analyses of giardial cells from trophozoites 12h, 24h encysting cells and complete cysts. RNA was extracted with Trizol reagent and cDNA was synthesized by RT-PCR. cDNA was analyzed using SYBR Green with the BioRad MyIQ system. Using various lipid genes shows that GlcT1 overexpression did not affect many lipid genes. The main lipid genes that were affected were the SPT genes where expression increased at 12h, but decreased at the cyst stage. In the other lipid genes group gFAELO in gGlcT1 overexpressed cells showed an increase in 12h as well, but a decrease in both 24h and cyst stages.

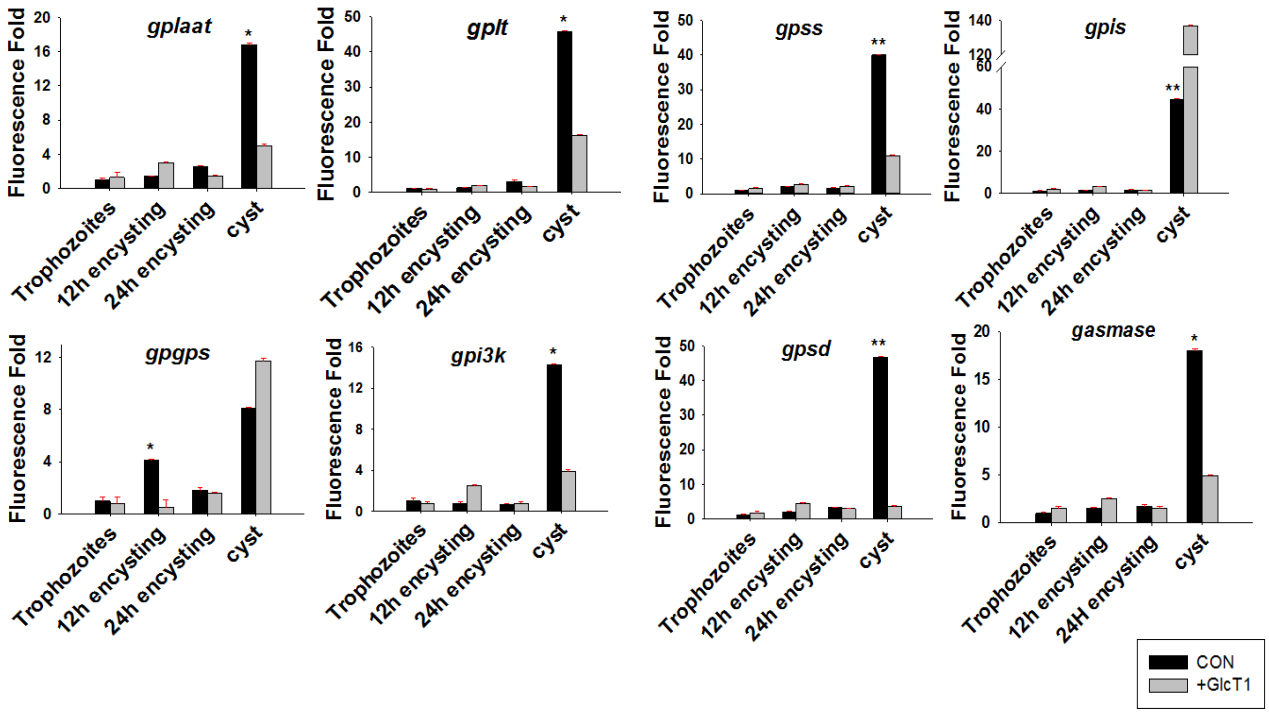

Figure 13: Real Time qRT-PCR Analysis of Lipid Genes in gGlcT1 Overexpressed *Giardia*

gPIS, the phospholipid gene showed over a 3-fold increase at the cyst stage during gGlcT1 overexpression. All other lipid profiles show only a decrease in the cyst stage meanwhile PGPS shows a slight increase in cyst stage, but a slight decrease in 12h encysting due to gGlcT1 overexpression.

gglct1, the expression of all lipid genes, investigated here are maximum in cysts. In gGlcT1-overexpressed cells, however, the transcripts of two serine-palmitoyltransferase genes (*gspt-1* and *gspt-2*) are increased ~10 fold and ~30 fold in 12h and 24h encysting cells, respectively, followed by a sharp decline in cysts. The giardial fatty acid elongase gene (*gfaelo*) showed a ~11-fold increase in expression at 12h encysting (**Fig 12**). Interestingly, the expression of acid sphingomyelinase gene (*gasmase*) remains unaltered throughout the encystation cycle in gGlcT1-overexpressed cells (**Fig 13**). As expected the synthesis of *gglct1* mRNA is increased ~9 and ~1.5 fold, respectively in 12 and 24h overexpressed encysting *Giardia*. On the contrary, the transcripts of other PL genes [e.g., phosphatidylinositol synthase (gPIS), phosphatidylglycerol synthase (gPGPS), phosphatidylserine synthase (gPSS), phospholipid transferase (gPLT), phosphatidylserine decarboxylase (gPSD), phospholipid transporting ATPase (gPLTA) and phosphatidylinositol-3-kinase (gPI3K) are down-regulated in cysts by gGlcT1 (**Fig. 13**).

3.2.3 gGlcT1 overexpression induces production of multi-layer ESVs

As previously shown, overexpression caused the synthesis of enlarged, aggregated ESVs under confocal microscopy using anti-cyst antibody. I decided to investigate using Transmission Electron Microscopy (TEM) to observe any structural differences of ESVs between wild-type and gGlcT1-overexpressed *Giardia* (**Fig 14**). Control ESVs show a uniform, thin-membrane layer surrounding the dense cyst wall material being transported to lay down the cyst wall. However, parasites that have gGlcT1 overexpressed, exhibit a vesicle that has enlarged, multi-layer membranes. In some ESVs, vacuole-like structures can be seen lining the surface of the ESV membrane whereas other ESVs has protrusions where thick membranes are entangled and aggregated around the cyst wall material. Therefore, along with the increase of fatty acid and cholesterol incorporation and internalization of fluorescent-labeled lipids, modulation of gGlcT1 changes the structural morphology of ESVs.

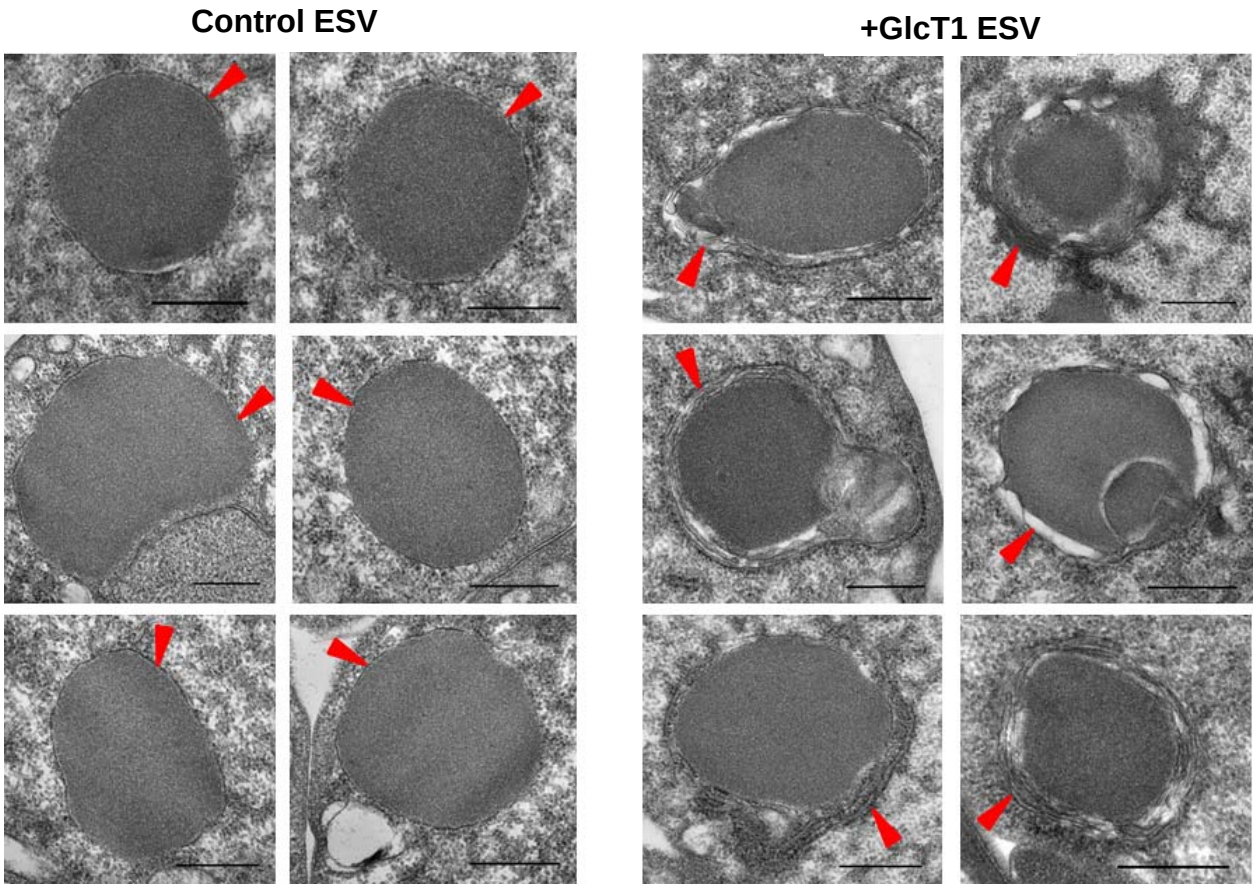

Figure 14: Analysis of ESV morphology under Transmission Electron Microscopy

Control and gGlcT1-overexpressed encysting *Giardia* were subjected to fixation, embedding and sectioning for electron microscopy. Several images of control show a thin bilayer surrounding the ESVs that transport cyst wall material to the plasma membrane. The ESVs themselves are overall round and uniform when compared to the +GlcT1 encysting cells which may show irregularities with the membrane. +GlcT1 encysting cells have multi-layer membranes that may contain vacuoles. The membranes of +GlcT1 ESVs also have tangled and enlarged membranes surrounding the cyst wall material. Bar: 0.5µm.

3.3 CONCLUSION

Because gGlcT1 overexpression induces the synthesis of enlarged ESVs during encystation, I thought that this could be due to the increased influx and utilization of exogenous lipids by the parasite. It was observed that gGlcT1 overexpression indeed elevated the levels of membrane lipids, cholesterol, and fatty acids. I presumed that this increased uptake of lipid molecules could be due to the changes of membrane fluidity/permeability caused by elevated gGlcT1 expression. However, when the membrane fluidity was tested, no significant effect was observed. In addition, qRT-PCR analysis indicated that the transcripts of only 3 genes (*gspt1*, *gspt2*, and *gfaelo*) are differentially expressed in gGlcT1 overexpressed trophozoites, which suggests that the effect of gGlcT1 overexpression is specific and selective and that this important enzyme of SL pathway plays a regulatory role during encystation at both the genetic and epigenetic levels. I speculate that the overexpression of gGlcT1 activates receptor-mediated endocytosis and transport proteins to internalize lipids and fatty acids by *Giardia*. However, more in-depth experiments should be carried out to test this possibility.

Chapter 4: Discussion

Giardia, an intestinal parasite, is known to cause gastrointestinal discomfort that could lead to symptoms like vomiting, diarrhea, indigestion, and abdominal cramps. Metronidazole, an analogue of nitroimidazole, was marketed in 1962 and used as an anti-giardial agent. Later on, albendazole and the furazolidone group of compounds were also introduced to treat the infection (Gardner et al. 2001). Unfortunately, many of these drugs are not specific for *Giardia* and may produce adverse side effects. Therefore, it is imperative to discover new targets that could be exploited to develop novel anti-giardial agents. In my dissertation, I have demonstrated that gGlcT1 regulates ESV biogenesis and cyst viability and that it may serve as a good candidate for drug targeting and vaccine development in the future.

In this dissertation, an attempt was made to elucidate the mechanism of action of gGlcT1 and how it regulates encystation and cyst production by *Giardia*. Analysis of the *Giardia* Genome Database (www.Giardiadb.org) revealed that only five SL metabolic genes (including *gglct1*) are present in this parasite and that all of these genes are differentially regulated during encystation (Hernandez et al. 2008). Interestingly, we and others have observed that PPMP, an inhibitor for GlcCer synthesis, blocks encystation and cyst production by *Giardia* (Hernandez et al. 2008; Sonda et al. 2008; Stefanic et al. 2010). Because earlier studies (Hernandez et al. 2008) from our laboratory indicated that gGlcT1 is the only SL biosynthesis enzyme that regulates the process of encystation in *Giardia*, I decided to study the mechanism by which gGlcT1 regulates the process of encystation. To initiate this, I standardized the assay of gGlcT1 following the protocols that were described for other organisms, with some essential modifications (Uchida et al. 2002; Couto et al. 2004). I found that the gGlcT1 activity increases during encystation and attains its highest activity level in cysts. To confirm the specificity of this enzyme, PPMP was added in the reaction mixture before determining the activity. It is of interest that PPMP, which inhibited the gGlcT1 activity in trophozoites and early encysting cells, had no effect on cyst enzyme (gGlcT1), suggesting that *Giardia* synthesizes PPMP-sensitive and -resistant gGlcT1. To further investigate this matter, the gglct1 gene was knocked down with anti-gglct1 morpholino oligonucleotide. **Figure 4B and 7A** demonstrate that the morpholino oligonucleotide lowered gGlcT1 activity significantly in non-encysting and encysting trophozoites as well as in cysts. Although it is not clear

why cyst-stage gGlcT1 is not inhibited by PPMP (**Fig. 4B**), Stefanic et al. (2010) reported that *Giardia* synthesizes di- and tri-hexosylceramides in encysting cells and cysts, indicating that gGlcT1 changes its substrate preferences during encystation and converts mono-hexosylceramide (i.e., GlcCer) to di- and tri-hexosylceramides. Hillig et al. (2003) reported that GlcT1 in plant cells shows broader substrate affinities and functions as a sterol glucoside synthase. Interestingly, GlcT1 in *Giardia* and plants are remarkably similar as far as the amino acid identity and sequence motifs are concerned, and therefore, like plant enzyme, gGlcT1 exhibits broader substrate preferences and catalyzes the synthesis of complex glycosylceramides, as shown in **Fig. 7C**. However, additional experiments are required to confirm this possibility.

gGlcT1 overexpression increased the activity of the enzyme by ~3 fold, induced enlarged/ aggregated ESVs, and produced non-viable cysts. However, when overexpressed trophozoites were transfected with anti-gglct1 morpholino oligonucleotide, the enzyme activity, ESV morphologies, and cyst viability returned to normal (**Fig 7A, 8 and 10**). Electron microscopy results showed that gGlcT1 overexpression also produced multi-layered ESVs, which is sometimes associated with vacuoles. Non-transfected encysting cells showed normal, bilayered membrane ESVs (**Fig 14**). A report of mink lung type II alveolar cells (Mv1Lu) transfected with β1–6-N-acetylglucosaminyl transferase V produced multi-lamellar vacuoles via autophagy. Upon treatment of the alveolar cells with a known inhibitor of autophagy, 3-methyladenine, the number of multi-lamellar vacuoles decreased (Hariri et al. 2000). Therefore, a similar kind of autophagic reaction may also occur in gGlcT1 overexpressed *Giardia* that induces the synthesis of enlarged and multi-layered ESVs during encystation. My results also showed that the overexpression of gGlcT1 alters the lipid balance by importing excess lipids, fatty acids, and cholesterol from the growth medium and that those lipids are most likely to be recruited by the cell to synthesize morphologically altered ESVs (**Fig. 11 and 14**). From these results, I postulate that gGlcT1 is part of a highly regulated pathway that plays a critical role in maintaining ESV biogenesis and cyst production and that it does so by maintaining the cellular lipid homeostasis (discussed in Chapter 3). Like *Giardia*, gGlcT1 overexpression also influences several body-fat storage genes in Drosophila

(Kohyama-Koganeya et al. 2011), which suggests that one of the functions of GlcT1 is to store lipids and maintain the overall lipid content for energy production and metabolic purposes.

Another important aspect of my thesis is identifying the fact that PPMP does not block giardial encystation by interfering with cytokinesis as previously reported. It was proposed that inhibiting the blocking of gGlcT1 activity interferes with cytokinesis and thereby obstructs encystation (Sonda et al. 2008; Stefanic et al. 2010). In the current study, however, it was noted that although the treatment of trophozoites with anti-gglct1 morpholino oligonucleotide inhibited gGlcT1 activity, it failed to affect the growth, replication, and cytokinesis of trophozoites (**Fig 9**). It is interesting that anti-gglct1 morpolino inhibited the biogenesis of ESVs and cyst production, which suggests that even though gGlcT1 activity is linked to encystation the blocking of trophozoite replication/cytokinesis by PPMP is an off-target effect and does not involve gGlcT1. It was shown earlier that PDMP, an analogue of PPMP, regulates the cell cycle by inhibiting cyclin-dependent kinases (cdk2 and p34cdc2), which further supports my results of non-specific inhibition by PPMP (Rani et al. 1995; Norris-Cervetto et al. 2004; Dijkhuis et al. 2006).

At this point, it is not yet clear how gGlcT1 regulates the cyst-wall biosynthesis. The rigid and rugged cyst wall of *Giardia* contains a fibrous and filamentous structure that is made of several cyst-wall proteins (CWP1, 2, and 3) and carbohydrates (mostly β-GalNAc homopolymer) (Gerwig et al. 2002; Chatterjee et al. 2010) It was proposed that CWP1 is a lectin that preferentially binds to GalNAc homopolymer to form a curled, fibril-type arrangement (Chatterjee et al. 2010). As far as GalNAc polymer is concerned, it forms flexible structures (right- or left-handed) with strong inter-chain interactions of an insoluble nature. Insoluble GalNAc homopolymer is synthesized from UDP-GalNAc with the help of an enzyme, called cyst-wall synthase (CWS, N-acetyl-galactosaminyltransferase) (Jarroll et al. 2001). It is of interest that so far no mechanism has been proposed to explain how the synthesis of cyst-wall components is initiated in the cytoplasm and transported to plasma membranes, and how the entire polymer coat is deposited on the outer face of the plasma membranes. In the case of cell-wall biosynthesis in gram-positive bacteria, it has been demonstrated that a complex saccharide- and peptide-containing lipid molecule (undecaprenyl disaccharide pentapeptide) acts as a carrier to transport

preformed peptidoglycan (synthesized in cytoplasm) to the plasma membrane to form cell walls (Navarre et al. 1999). I have demonstrated that gGlcT1 activity is increased during encystation (**Fig. 4A**) and that the relative abundances of mono-, di-, and tri-hexosylceramide are higher in encysting cells than non-encysting trophozoites (**Fig. 7C**). Therefore, it is likely that the formation of hexosylceramides in encysting cells initiates and transports cyst-wall components across the plasma membranes and lays down the cyst wall. It is also possible that hexosylceramides are present in the cyst wall and that their interactions with proteins and amino sugars (present in the cyst wall) are important for maintaining the integrity of cysts. It is also possible that gGlcT1 interacts with CWS to facilitate the encystation process. However, additional experiments are required to test these possibilities.

4.1 PROPOSED MODEL

Based on my results, I have inferred a comprehensive model describing how gGlcT1 modulates ESV biogenesis and cyst production (**Fig. 15**). The model reveals that the activity gGlcT1 enzyme (located in the ER) is stimulated during encystation and facilitates the synthesis of GlcCer from ceramide. Because *Giardia* is unable to synthesize its own lipids, including ceramide, it is taken up by *Giardia* from the culture medium via clathrin-dependent endocytosis, targeted toward the ER, and used as a substrate for gGlcT1 (Hernandez et al. 2007; Hernandez et al. 2008). Apparently, this reaction takes place in the ER, which is also the site of ESV biogenesis. GlcCer and other complex ceramides synthesized by the gGlcT1 reaction then participate in ESV biogenesis. My results also suggest that gGlcT1, through an unknown mechanism, regulates lipid and fatty acid uptake from the environment. As reviewed before (Yichoy et al. 2011), *Giardia* synthesizes putative phospholipid transporting ATPases (flippases), fatty acid transporters, and clathrin-coated vesicles to transport lipid and fatty acids from the cell exterior. It is possible that these exogenous lipids are used to synthesize membrane-bound ESVs and that this entire process is under the control of gGlcT1. When the enzyme is overexpressed, it severely alters the lipid internalization and affects the overall lipid homeostasis. Excess lipids then interfere with normal ESV synthesis and produce enlarged and multi-layered ESVs that are detrimental to viable cyst production.

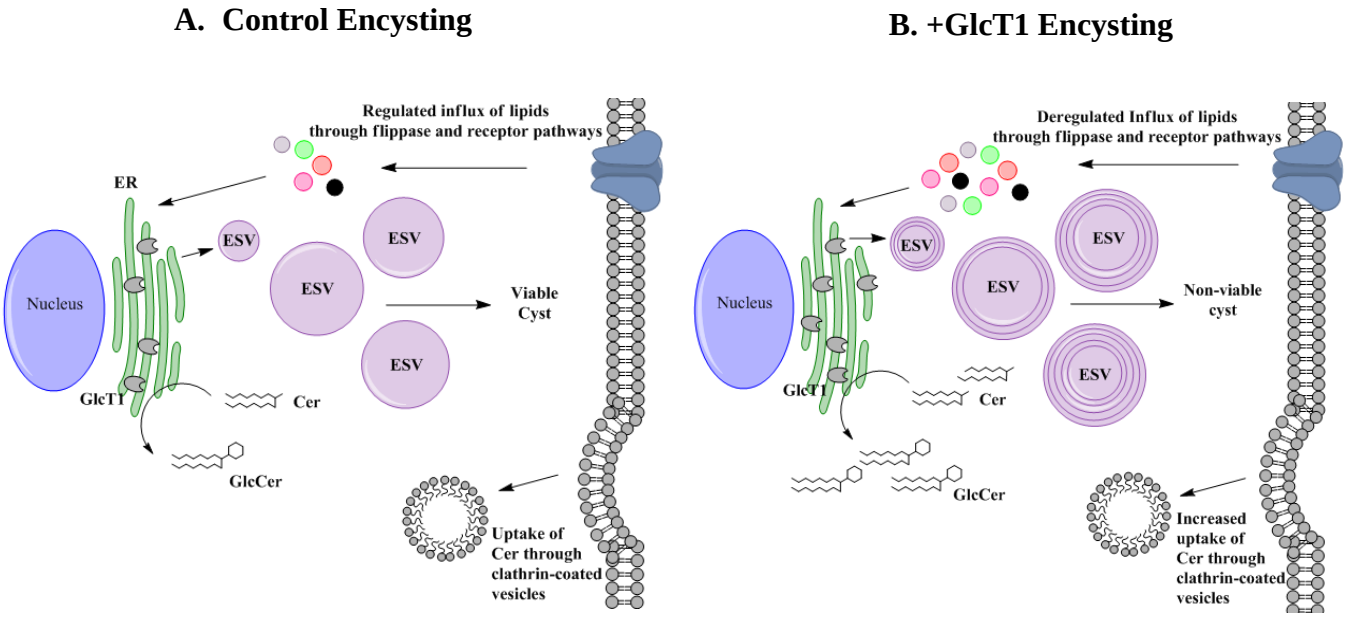

Figure 15: Proposed model of Giardial encystation pathway

Based on the results from this thesis, we have compiled an encystation pathway which shows (A) control cells and (B) gGlcT1-overexpressed cells.

4.2 CONCLUSION AND FUTURE DIRECTIONS

During its colonization in the human small intestine, *Giardia* is exposed to various dietary lipids. These intestinal lipids facilitate the growth and encystation of this waterborne pathogen. Encystation and cyst formation are important because they allow the parasite to survive in the environment and infect new hosts. My results show that the regulated expression of gGlcT1 activity is critical for cyst formation. In addition, my study indicates that gGlcT1 activity increases during encystation and is highest in cyst. Consistent with this result, I have also observed that di- and tri-hexosylceramides are synthesized during encystation, implicating a possible link between cyst-specific gGlcT1 and hexosylceramide syntheses. The future study will reveal the unique role of gGlcT1 and its product(s) in cyst formation. If successful, the study will set the stage for future investigations to develop potential therapies targeting glycospingolipids in *Giardia*. In recent years, efforts have been underway to produce vaccine against *Giardia* trophozoites (Rivero et al. 2010). The results generated from this dissertation should provide valuable information about whether gGlcT1 knockdown-attenuated cysts with reduced viability could be utilized as a live vaccine candidate against giardiasis in the future.

References

Adam, R. D. (2001). "Biology of Giardia lamblia." Clinical microbiology reviews **14**(3): 447-475.

Applebee, A., Thorlakson, C. & Olson M.E. (2002). Giardia: The Cosmopolitan Parasite, CABI.

Baldursson, S. and P. Karanis (2011). "Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010." Water Res **45**(20): 6603-6614.

Bektas, M., et al. (2010). "Sphingosine 1-phosphate lyase deficiency disrupts lipid homeostasis in liver." The Journal of biological chemistry **285**(14): 10880-10889.

Bernander, R., et al. (2001). "Genome ploidy in different stages of the Giardia lamblia life cycle." Cell Microbiol **3**(1): 55-62.

Bingham, A. K. and E. A. Meyer (1979). "Giardia excystation can be induced in vitro in acidic solutions." Nature **277**(5694): 301-302.

Bittencourt-Silvestre, J., et al. (2010). "Encystation process of Giardia lamblia: morphological and regulatory aspects." Arch Microbiol **192**(4): 259-265.

Blair, R. J. and P. F. Weller (1987). "Uptake and esterification of arachidonic acid by trophozoites of *Giardia lamblia*." Molecular and Biochemical Parasitology **25**(1): 11-18.

Boucher, S. E. and F. D. Gillin (1990). "Excystation of in vitro-derived Giardia lamblia cysts." Infection and immunity **58**(11): 3516-3522.

Carpenter, M. L., et al. (2012). "Nuclear inheritance and genetic exchange without meiosis in the binucleate parasite Giardia intestinalis." J Cell Sci **125**(Pt 10): 2523-2532.

Carpenter, M. L. and W. Z. Cande (2009). "Using morpholinos for gene knockdown in Giardia intestinalis." Eukaryot Cell **8**(6): 916-919.

Castillo-Romero, A., et al. (2010). "Rab11 and actin cytoskeleton participate in Giardia lamblia encystation, guiding the specific vesicles to the cyst wall." PLoS neglected tropical diseases **4**(6): e697.

Chatterjee, A., et al. (2010). "Giardia cyst wall protein 1 is a lectin that binds to curled fibrils of the GalNAc homopolymer." PLoS pathogens **6**(8).

Ciucanu, I., Kerek. FJ (1984). "A simple and rapid method for the permethylation of carbohydrate." Carbohydrate Research (131): 209-217.

Couto, A. S., et al. (2004). "Glycosphingolipids in Plasmodium falciparum. Presence of an active glucosylceramide synthase." European journal of biochemistry / FEBS **271**(11): 2204-2214.

Cox, S. S., et al. (2006). "Evidence from bioinformatics, expression and inhibition studies of phosphoinositide-3 kinase signalling in Giardia intestinalis." BMC Microbiol **6**: 45.

Das, S., et al. (2001). "Phospholipid remodeling/generation in Giardia: the role of the Lands cycle." Trends in parasitology **17**(7): 316-319.

Das, S., et al. (1988). "Killing of Giardia lamblia trophozoites by human intestinal fluid in vitro." J Infect Dis **157**(6): 1257-1260.

Das, S., et al. (1997). "Giardia lamblia: evidence for carrier-mediated uptake and release of conjugated bile acids." Experimental parasitology **87**(2): 133-141.

Das, S., et al. (1991). "A surface antigen of Giardia lamblia with a glycosylphosphatidylinositol anchor." The Journal of biological chemistry **266**(31): 21318-21325.

Diamond, L. S., et al. (1978). "A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba." Trans R Soc Trop Med Hyg **72**(4): 431-432.

Dijkhuis, A. J., et al. (2006). "PDMP sensitizes neuroblastoma to paclitaxel by inducing aberrant cell cycle progression leading to hyperploidy." Mol Cancer Ther **5**(3): 593-601.

DuBois, K. N., et al. (2008). "Identification of the major cysteine protease of Giardia and its role in encystation." The Journal of biological chemistry **283**(26): 18024-18031.

Ellis, J. E., et al. (1996). "Changes in lipid composition during in vitro encystation and fatty acid desaturase activity of Giardia lamblia." Molecular and biochemical parasitology **81**(1): 13-25.
Faso, C. and A. B. Hehl (2011). "Membrane trafficking and organelle biogenesis in Giardia lamblia: use it or lose it." International journal for parasitology **41**(5): 471-480.
Fridberg, A., et al. (2008). "Sphingolipid synthesis is necessary for kinetoplast segregation and cytokinesis in Trypanosoma brucei." J Cell Sci **121**(Pt 4): 522-535.
Gardner, T. B. and D. R. Hill (2001). "Treatment of giardiasis." Clinical microbiology reviews **14**(1): 114-128.
Gerwig, G. J., et al. (2002). "The Giardia intestinalis filamentous cyst wall contains a novel beta(1-3)-N-acetyl-D-galactosamine polymer: a structural and conformational study." Glycobiology **12**(8): 499-505.
Gibson, G. R., et al. (1999). "Giardia lamblia: incorporation of free and conjugated fatty acids into glycerol-based phospholipids." Experimental parasitology **92**(1): 1-11.
Gillin, F. D. (1987). "Giardia lamblia: the role of conjugated and unconjugated bile salts in killing by human milk." Experimental parasitology **63**(1): 74-83.
Gillin, F. D., et al. (1989). "Giardia lamblia: the roles of bile, lactic acid, and pH in the completion of the life cycle in vitro." Experimental parasitology **69**(2): 164-174.
Gillin, F. D., et al. (1988). "Small-intestinal factors promote encystation of Giardia lamblia in vitro." Infection and immunity **56**(3): 705-707.
Gupta, V., et al. (2010). "Direct quantitative determination of ceramide glycosylation in vivo: a new approach to evaluate cellular enzyme activity of glucosylceramide synthase." Journal of lipid research **51**(4): 866-874.
Hanada, K. (2005). "Sphingolipids in infectious diseases." Japanese journal of infectious diseases **58**(3): 131-148.
Hariri, M., et al. (2000). "Biogenesis of multilamellar bodies via autophagy." Mol Biol Cell **11**(1): 255-268.
Hehl, A. B. and M. Marti (2004). "Secretory protein trafficking in Giardia intestinalis." Molecular microbiology **53**(1): 19-28.
Hermansson, M., et al. (2011). "Mechanisms of glycerophospholipid homeostasis in mammalian cells." Prog Lipid Res **50**(3): 240-257.
Hernandez, Y., et al. (2007). "Clathrin-dependent pathways and the cytoskeleton network are involved in ceramide endocytosis by a parasitic protozoan, Giardia lamblia." International journal for parasitology **37**(1): 21-32.
Hernandez, Y., et al. (2008). "Novel role of sphingolipid synthesis genes in regulating giardial encystation." Infection and immunity **76**(7): 2939-2949.
Hernandez, Y., et al. (2007). "Transcriptional analysis of three major putative phosphatidylinositol kinase genes in a parasitic protozoan, Giardia lamblia." The Journal of eukaryotic microbiology **54**(1): 29-32.
Hillig, I., et al. (2003). "Formation of glucosylceramide and sterol glucoside by a UDP-glucose-dependent glucosylceramide synthase from cotton expressed in Pichia pastoris." FEBS letters **553**(3): 365-369.
Hopkins, R. M., et al. (1997). "Ribosomal RNA sequencing reveals differences between the genotypes of Giardia isolates recovered from humans and dogs living in the same locality." J Parasitol **83**(1): 44-51.
House, S. A., et al. (2011). "Giardia flagellar motility is not directly required to maintain attachment to surfaces." PLoS pathogens **7**(8): e1002167.
Huang, Y. C., et al. (2008). "Regulation of cyst wall protein promoters by Myb2 in Giardia lamblia." The Journal of biological chemistry **283**(45): 31021-31029.

Jarroll, E. L., et al. (2001). "Regulation of carbohydrate metabolism during Giardia encystment." J Eukaryot Microbiol **48**(1): 22-26.
Jarroll, E. L., et al. (1981). "Lipid and carbohydrate metabolism of Giardia lamblia." Molecular and biochemical parasitology **2**(3-4): 187-196.
Kane, A. V., et al. (1991). "In vitro encystation of Giardia lamblia: large-scale production of in vitro cysts and strain and clone differences in encystation efficiency." J Parasitol **77**(6): 974-981.
Kaneda, Y. and T. Goutsu (1988). "Lipid analysis of Giardia lamblia and its culture medium." Ann Trop Med Parasitol **82**(1): 83-90.
Keister, D. B. (1983). "Axenic culture of Giardia lamblia in TYI-S-33 medium supplemented with bile." Trans R Soc Trop Med Hyg **77**(4): 487-488.
Kohyama-Koganeya, A., et al. (2011). "Glucosylceramide synthase in the fat body controls energy metabolism in Drosophila." Journal of lipid research **52**(7): 1392-1399.
Kovacs, P., et al. (2000). "Effect of glucosphingolipid synthesis inhibitor (PPMP and PDMP) treatment on Tetrahymena pyriformis: data on the evolution of the signaling system." Cell Biochem Funct **18**(4): 269-280.
Kulda J, N. E. (1978). "Giardia and Giardiasis." Parasitic Protozoa: 69-138.
Lane, S. and D. Lloyd (2002). "Current trends in research into the waterborne parasite Giardia." Crit Rev Microbiol **28**(2): 123-147.
Lanfredi-Rangel, A., et al. (2003). "Fine structure of the biogenesis of Giardia lamblia encystation secretory vesicles." J Struct Biol **143**(2): 153-163.
Lasek-Nesselquist, E., et al. (2010). "The identification of a new Giardia duodenalis assemblage in marine vertebrates and a preliminary analysis of G. duodenalis population biology in marine systems." International journal for parasitology **40**(9): 1063-1074.
Lauwaet, T., et al. (2007). "Encystation of Giardia lamblia: a model for other parasites." Current opinion in microbiology **10**(6): 554-559.
Li, Y., et al. (2008). "Sensitive detection of isoglobo and globo series tetraglycosylceramides in human thymus by ion trap mass spectrometry." Glycobiology **18**(2): 158-165.
Lindmark, D. G. (1980). "Energy metabolism of the anaerobic protozoon Giardia lamblia." Molecular and biochemical parasitology **1**(1): 1-12.
Lujan, H. D., et al. (1996). "Cholesterol starvation induces differentiation of the intestinal parasite Giardia lamblia." Proc Natl Acad Sci U S A **93**(15): 7628-7633.
Lujan, H. D., et al. (1995). "Isoprenylation of proteins in the protozoan *Giardia lamblia*." Molecular and Biochemical Parasitology **72**(1-2): 121-127.
Lujan, H. D., et al. (1996). "Lipid requirements and lipid uptake by Giardia lamblia trophozoites in culture." The Journal of eukaryotic microbiology **43**(3): 237-242.
Maldonado, R. A., et al. (2006). "Trypanosoma cruzi oleate desaturase: molecular characterization and comparative analysis in other trypanosomatids." J Parasitol **92**(5): 1064-1074.
Mayrhofer, G., et al. (1995). "Division of Giardia isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with Giardia muris." Parasitology **111 (Pt 1)**: 11-17.
McCaffery, J. M. and F. D. Gillin (1994). "Giardia lamblia: ultrastructural basis of protein transport during growth and encystation." Experimental parasitology **79**(3): 220-235.
Melser, S., et al. (2010). "Glucosylceramide biosynthesis is involved in Golgi morphology and protein secretion in plant cells." Traffic **11**(4): 479-490.
Mendis, A. H., et al. (1992). "The uptake and conversion of L-[U14C-] aspartate and L-[U14C-] alanine to 14CO2 by intact trophozoites of Giardia duodenalis." Comp Biochem Physiol B **102**(2): 235-239.

Mohareb, E. W., et al. (1991). "*Giardia lamblia*: phospholipid analysis of human isolates." Annals of Tropical Medicine and Parasitology **85**(6): 591-597.
Morgan, C. P., et al. (2004). "Identification of phospholipase B from Dictyostelium discoideum reveals a new lipase family present in mammals, flies and nematodes, but not yeast." The Biochemical journal **382**(Pt 2): 441-449.
Morrison, H. G., et al. (2007). "Genomic minimalism in the early diverging intestinal parasite Giardia lamblia." Science **317**(5846): 1921-1926.
Morrison, H. G., et al. (2002). "Inferring protein function from genomic sequence: Giardia lamblia expresses a phosphatidylinositol kinase-related kinase similar to yeast and mammalian TOR." Comp Biochem Physiol B Biochem Mol Biol **133**(4): 477-491.
Natoli, T. A., et al. (2010). "Inhibition of glucosylceramide accumulation results in effective blockade of polycystic kidney disease in mouse models." Nat Med **16**(7): 788-792.
Navarre, W. W. and O. Schneewind (1999). "Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope." Microbiol Mol Biol Rev **63**(1): 174-229.
Norris-Cervetto, E., et al. (2004). "Inhibition of glucosylceramide synthase does not reverse drug resistance in cancer cells." J Biol Chem **279**(39): 40412-40418.
O'Handley, R. M., et al. (2000). "Prevalence and genotypic characterisation of Giardia in dairy calves from Western Australia and Western Canada." Vet Parasitol **90**(3): 193-200.
Ortega, Y. R. and R. D. Adam (1997). "Giardia: overview and update." Clin Infect Dis **25**(3): 545-549; quiz 550.
Pan, Y. J., et al. (2009). "A novel WRKY-like protein involved in transcriptional activation of cyst wall protein genes in Giardia lamblia." The Journal of biological chemistry **284**(27): 17975-17988.
Pernet, F., et al. (2006). "Comparison of three solid-phase extraction methods for fatty acid analysis of lipid fractions in tissues of marine bivalves." J Chromatogr A **1137**(2): 127-137.
Pewzner-Jung, Y., et al. (2010). "A critical role for ceramide synthase 2 in liver homeostasis: I. alterations in lipid metabolic pathways." The Journal of biological chemistry **285**(14): 10902-10910.
Poxleitner, M. K., et al. (2008). "Evidence for karyogamy and exchange of genetic material in the binucleate intestinal parasite Giardia intestinalis." Science **319**(5869): 1530-1533.
Rani, C. S., et al. (1995). "Cell cycle arrest induced by an inhibitor of glucosylceramide synthase. Correlation with cyclin-dependent kinases." The Journal of biological chemistry **270**(6): 2859-2867.
Rayan, P., et al. (2005). "The effects of saturated fatty acids on Giardia duodenalis trophozoites in vitro." Parasitology research **97**(3): 191-200.
Regoes, A., et al. (2005). "Protein import, replication, and inheritance of a vestigial mitochondrion." The Journal of biological chemistry **280**(34): 30557-30563.
Reiner, D. S., et al. (2008). "Synchronisation of Giardia lamblia: identification of cell cycle stage-specific genes and a differentiation restriction point." International journal for parasitology **38**(8-9): 935-944.
Reiner, D. S., et al. (1990). "Sorting of cyst wall proteins to a regulated secretory pathway during differentiation of the primitive eukaryote, Giardia lamblia." Eur J Cell Biol **53**(1): 142-153.
Reiner, D. S., et al. (1986). "Human milk kills Giardia lamblia by generating toxic lipolytic products." J Infect Dis **154**(5): 825-832.
Rivero, F. D., et al. (2010). "Disruption of antigenic variation is crucial for effective parasite vaccine." Nat Med **16**(5): 551-557, 551p following 557.
Rivero, M. R., et al. (2010). "Long double-stranded RNA produces specific gene downregulation in Giardia lamblia." J Parasitol **96**(4): 815-819.

Sackey, M. E., et al. (2003). "Predictors and nutritional consequences of intestinal parasitic infections in rural Ecuadorian children." J Trop Pediatr **49**(1): 17-23.
Schofield, P. J., et al. (1995). "Amino acid exchange activity of the alanine transporter of Giardia intestinalis." Experimental parasitology **80**(1): 124-132.
Schofield, P. J., et al. (1992). "The pathway of arginine catabolism in Giardia intestinalis." Molecular and biochemical parasitology **51**(1): 29-36.
Schupp, D. G. and S. L. Erlandsen (1987). "Determination of Giardia muris cyst viability by differential interference contrast, phase, or brightfield microscopy." J Parasitol **73**(4): 723-729.
Schupp, D. G. and S. L. Erlandsen (1987). "A new method to determine Giardia cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity." Appl Environ Microbiol **53**(4): 704-707.
Sener, K., et al. (2004). "Amino sugar phosphate levels in Giardia change during cyst wall formation." Microbiology **150**(Pt 5): 1225-1230.
Shukla, A. and N. S. Radin (1991). "Metabolism of D-[3H]threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthesis, and the synergistic action of an inhibitor of microsomal monooxygenase." Journal of lipid research **32**(4): 713-722.
Slavin, I., et al. (2002). "Dephosphorylation of cyst wall proteins by a secreted lysosomal acid phosphatase is essential for excystation of Giardia lamblia." Molecular and biochemical parasitology **122**(1): 95-98.
Sonda, S., et al. (2008). "A sphingolipid inhibitor induces a cytokinesis arrest and blocks stage differentiation in Giardia lamblia." Antimicrobial agents and chemotherapy **52**(2): 563-569.
Stefanic, S., et al. (2009). "Neogenesis and maturation of transient Golgi-like cisternae in a simple eukaryote." J Cell Sci **122**(Pt 16): 2846-2856.
Stefanic, S., et al. (2010). "Glucosylceramide synthesis inhibition affects cell cycle progression, membrane trafficking, and stage differentiation in Giardia lamblia." Journal of lipid research **51**(9): 2527-2545.
Stevens, T. L., et al. (1997). "Uptake and cellular localization of exogenous lipids by Giardia lamblia, a primitive eukaryote." Experimental parasitology **86**(2): 133-143.
Subramanian, A. B., et al. (2000). "Role of exogenous inositol and phosphatidylinositol in glycosylphosphatidylinositol anchor synthesis of GP49 by Giardia lamblia." Biochimica et biophysica acta **1483**(1): 69-80.
Sun, Y. L., et al. (2006). "Suppression of glucosylceramide synthase by RNA interference reverses multidrug resistance in human breast cancer cells." Neoplasma **53**(1): 1-8.
Talal, A. H. and J. A. Murray (1994). "Acute and chronic diarrhea. How to keep laboratory testing to a minimum." Postgrad Med **96**(3): 30-32, 35-38, 43 passim.
Thevissen, K., et al. (2004). "Defensins from insects and plants interact with fungal glucosylceramides." The Journal of biological chemistry **279**(6): 3900-3905.
Touz, M. C., et al. (2005). "A novel palmitoyl acyl transferase controls surface protein palmitoylation and cytotoxicity in Giardia lamblia." Molecular microbiology **58**(4): 999-1011.
Touz, M. C., et al. (2003). "Sorting of encystation-specific cysteine protease to lysosome-like peripheral vacuoles in Giardia lamblia requires a conserved tyrosine-based motif." The Journal of biological chemistry **278**(8): 6420-6426.
Uchida, Y., et al. (2002). "Glucosylceramide synthesis and synthase expression protect against ceramide-induced stress." Journal of lipid research **43**(8): 1293-1302.
Vargas-Villarreal, J., et al. (2007). "Activity of intracellular phospholipase A1 and A2 in *Giardia lamblia*." Journal of Parasitology **93**(5): 979-984.
Ward, W., et al. (1997). "A primitive enzyme for a primitive cell: the protease required for excystation of Giardia." Cell **89**(3): 437-444.

Wolfe, M. S. (1992). "Giardiasis." Clinical microbiology reviews **5**(1): 93-100.
Worgall, T. S., et al. (2004). "Sterol and fatty acid regulatory pathways in a Giardia lamblia-derived promoter: evidence for SREBP as an ancient transcription factor." Journal of lipid research **45**(5): 981-988.
Wright, S. G. (2012). "Protozoan infections of the gastrointestinal tract." Infect Dis Clin North Am **26**(2): 323-339.
Yew, N. S., et al. (2010). "Increased hepatic insulin action in diet-induced obese mice following inhibition of glucosylceramide synthase." PLoS One **5**(6): e11239.
Yichoy, M., et al. (2011). "Lipid metabolism in Giardia: a post-genomic perspective." Parasitology **138**(3): 267-278.
Yichoy, M., et al. (2009). "Lipidomic analysis reveals that phosphatidylglycerol and phosphatidylethanolamine are newly generated phospholipids in an early-divergent protozoan, Giardia lamblia." Molecular and biochemical parasitology **165**(1): 67-78.
Yoder, J. S., et al. (2010). "Giardiasis surveillance - United States, 2006-2008." MMWR Surveill Summ **59**(6): 15-25.

Curriculum Vita

Tavis began his college career with a Bachelor's of Science in Microbiology with a minor in Chemistry in the summer of 2002 at the University of Texas at El Paso. He began to work with Dr. Das in 2003 and finished his undergraduate degree in 2007. During his time as an undergraduate, Tavis also had an opportunity to conduct research in Nanjing, China with Dr. Shengnan Li at Nanjing Medical University for the summer of 2005. Tavis continued to work with Dr. Das with sphingolipids and *Giardia lamblia* under the doctoral program. He has attended several meetings including Molecular Parasitology Meeting in Woods Hole, MA in 2007, The International Conference of Anaerobic Parasites in Los Angeles, CA in 2012, and the Rio Grande Branch American Society for Microbiology Meeting at Socorro, NM in 2013. Moreover, Tavis has been a recipient of awards such as the RISE fellowship and Dodson fellowship. Tavis' dissertation, "*Giardia lamblia*: Regulation of Cyst Production by Glycosphingolipids" was supervised by Professor Siddhartha Das.

Permanent Address:
4-13-20, Room No. 402
Shiratori, Katsushika-ku
Tokyo, Japan 125-0063