Molecular and Cellular Characterizations of Glucosylceramide Transferase (Synthase) in Giardia lamblia

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MOLECULAR AND CELLULAR CHARACTERIZATIONS OF
GLUCOSYLCERAMIDE TRANSFERASE
(SYNTHASE) IN GIARDIA LAMBLIA

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I dedicate this to my family, who instilled in me the commitment and
drive for learning, curiosity, and perseverance.
THE MOLECULAR AND CELLULAR CHARACTERIZATIONS OF
GLUCOSYLCERAMIDE TRANSFERASE
(SYNTHASE) IN GIARDIA LAMBLIA

By

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DISSERTATION

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The University of Texas at El Paso
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ABSTRACT

Giardiasis, caused by an intestinal protozoan, *Giardia lamblia*, is a major public health problem worldwide. *Giardia* infection is spread through the fecal-oral route via contaminated water and food. It is estimated that over 280 million people are infected with *Giardia* across the globe. *Giardia* is a non-invasive parasite and consists of a two-stage life cycle: trophozoites and cysts. Ingested cysts undergo excystation in the proximal small intestine, releasing two trophozoites (excystoites). Upon reaching the distal small intestine and colonizing there, trophozoites transform into cysts by encystation. It has been reported earlier that the process of encystation induced the synthesis of giardial glucosylceramide transferase-1 (gGlcT1), an enzyme of the sphingolipid pathway that participates in various biological processes including cell division, cytokinesis, ESV biogenesis, cyst production, and maintaining the cellular lipid homeostasis. The modulation of its activity using molecular tools (overexpression, knockdown and rescue) interfered with cyst production, cyst viability, and infectivity in laboratory animals. gGlcT1 is unique because it is sufficiently diverged from other eukaryotic GlcT enzymes and consists of two active catalytic domains (CGlcT and CGalT) that catalyze the synthesis of glucosylceramide (GlcCer) and galactosylceramide (GalCer), respectively in a non-competitive manner.

The goal of my dissertation is to further analyze these two catalytic domains and elucidate their roles in growth, encystation and cyst production by *Giardia*. To address this goal, I used homologous recombination and overexpression techniques and created deletion mutant *Giardia* cell lines. I found that both catalytic domains are active and participate in cyst production and maintaining cyst wall structures. I also observed that
*Giardia* expresses a long-chain fatty acid elongase (gFAELO) enzyme, which is involved in the remodeling of membrane lipids by exchanging fatty acyl moieties (the Land’s cycle). Since gFAELO is mostly expressed in cysts and gGlcT1 overexpression affects lipid uptake, I investigated whether the interplay between gGlcT1 and gFAELO is important for cyst production, morphology, and viability as these events dictate the parasite’s ability to transmit the disease process. Therefore, the goal of my project was to elucidate the molecular mechanisms behind gGlcT1 and gFAELO as they pertain to the life cycle of *Giardia*. I hypothesize that The Specific Aims of the project were to: (1) use molecular techniques to determine which domain/s of gGlcT1 were important for its function; (2) and to investigate the how the domains of gGlcT1 and its interaction with gFAELO would modulate cyst morphology. *I hypothesize that elucidating the function of glucosylceramide transferase will reveal the mechanisms of giardial differentiation and could be exploited to design novel drugs against this pathogen*. Therefore, The Specific Aims of the project are to: (1) use molecular techniques to determine which domain/s of gGlcT1 were important for its function; (2) and to investigate the how the domains of gGlcT1 and its interaction with gFAELO would modulate cyst morphology. It is anticipated that the findings of this study will elucidate new knowledge about gGlcT1 and gFAELO interplay and the mechanisms of cyst production by *Giardia*. 
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Notation regarding nomenclature: This dissertation refers to gene names in several contexts. Nomenclature by gene and protein is as follows.

Giardia Glucosylceramide Transferase Gene: lowercase italics-gg1ct1
Giardia Glucosylceramide Transferase Protein: without italics-gGlCt1
Giardia Fatty Acid Elongase 1 Gene: lowercase italics-gfao
Giardia Fatty Acid Elongase 1 Protein: without italics-gFAELO
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1. INTRODUCTION

1.1 *Giardia* and *Giardiasis*

Globally, diarrhea is the leading cause of human morbidity and mortality second to lower respiratory infections (1). There are an estimated 1.5 million deaths recorded annually, with the majority being children (2). Diarrhea is also the second most cause of death in children worldwide (3). In a global review of diarrheal disease in children, the most common etiological agents were found to be viral (e.g., rotavirus, calicivirus, adenovirus), bacterial (e.g., ETEC, *Shigella spp*, *Salmonella spp*), or parasitic pathogens (i.e., *Cryptosporidium spp*, *Giardia lamblia*, or *Entamoeba histolytica*) (4). There is an established link between socioeconomic status and public health (5). A long-standing example of this is how diarrheal illnesses play a prominent role in the morbidity and mortality of children living in developing countries (6). In a multivariate study on a cohort of children living in Peru, wealth displayed a negative correlation with giardial infection (7). In other words, the diarrheal illness giardiasis was more strongly associated with low-income neighborhoods. Moreover, diarrheal illnesses can be associated with other risk factors such as access to clean water sources, sanitation, and nourishment (7). Even though these diseases can be self-limiting, children are at a disadvantage from recovering due to the aforementioned risk factors, making the causes of diarrhea important public health and health disparity issues to address.

The zoonotic and waterborne diarrheal illness known as giardiasis is caused by the protozoan intestinal parasite *Giardia lamblia*. Giardiasis is one of the most diagnosed diarrheal illnesses worldwide, with around 280 million cases diagnosed per year (8). In 2012, about 15,000 cases of giardiasis were reported in the U.S. alone, with one third
being hospitalized. Like other common diarrheal diseases, children under the age of 5 are disproportionately affected as seen in Figure 1. To further emphasize the prevalence of this disease, it has been noted that children in developing countries have been infected with giardiasis at least once in their lifetime (9). The infection could be either symptomatic or asymptomatic (10). Acute symptoms of giardiasis include severe diarrhea, dehydration, greasy stool, and abdominal cramps. Chronic symptoms can range from failure to thrive, cognitive impairment, and arrested growth and development in children, as well as irritable bowel syndrome in adults (11).

Figure 1. Annual number of cases and incidence rate of giardiasis by age in 2011-2012. The annual number of cases and the incidence rate of giardiasis in 2011-2012 is the highest in children under the age of 5 compared to the rest of the population (12). Figure adapted from the CDC.

The parasite is transmitted through food, water, and feces contaminated with giardial cysts (10). It has a simple life cycle composed of two life stages as seen in Figure
2: a binucleated, anaerobic teardrop-shaped trophozoite and an osmotically-resistant, “dormant” oval-shaped cyst. Once ingested, the cyst transforms into trophozoites by the process known as excystation. The trophozoites colonize the upper small intestine and transform into cysts in the lower small intestine by encystation (10). The infectious cyst is then transmitted in the environment and the cycle repeats (Figure 2).

In vitro encystation methods have made it possible to investigate encystation more in detail (13,14). Part of the revelations associated with the encystation process are the study of encystation-specific vesicles or ESVs. These vesicles are a part of a unique secretory system in *Giardia*, which is considered a regulated pseudo-Golgi. The transport and formation of these vesicles is a tightly regulated occurrence during encystation. These vesicles are secreted and fuse to the plasma membrane to deliver Cyst Wall Proteins (CWPs) and other cyst wall components (Figure 3) (15,16). For this reason, ESVs are an important factor to consider when studying the parasite’s ability to differentiate and generate infectious cysts.

*Giardia* is often a topic of interest because it is considered one of the earliest unicellular eukaryotic cells and therefore serves as a model organism to study the origin and evolution of life (17). The mechanism of pathogenesis utilized by Giardia leads to the damaging of the intestinal mucosa and generating inflammatory responses (10). Since the parasite has limited *de novo* lipid synthesis ability, it uptakes lipids from the hosts and the environment for survival, replication, and encystation (17). However, the orchestrated molecular mechanisms of transforming from trophozoite to cyst form (i.e., encystation) or cysts to trophozoites (e.g., excystation) remain enigmatic.
Figure 2. The simple dual life cycle of *Giardia lamblia*. *Giardia* has two life stages that are the trophozoite and cyst form (9). A person contracts the disease by ingesting contaminated food or water with cysts. The cysts excyst in the GI tract giving rise to two trophozoites. These colonize the small intestine and absorb nutrients in the intestinal milieu. In the distal small intestine, trophozoites encyst into the cyst form and are released into the environment through stool and the cycle is left to repeat. Figure adapted from the CDC.
Figure 3. **The process of encystation or the transition of trophozoites to cysts.** Panels A-D demonstrate DIC images of encystation, while panels E-H show the localization of cyst wall proteins during encystation. As seen in panel F, CWPs are localized to the encystation specific vesicles (ESVs) and migrate to the cell’s periphery where they are integrated into the cyst wall. The ESVs carry other necessary components of the cyst wall during encystation (15). Figure adapted from Mendez et al. (2015).

1.2 **The Sphingolipid Pathway in Giardia**

Our laboratory, for the past several years is investigating the role of sphingolipids (SLs) in regulating the encystation and cyst formation by *Giardia*. Sphingolipids (SLs) are both important structural components of membranes and active signaling molecules that are implicated in various cellular processes such as growth, differentiation, raft assembly, apoptosis, senescence, and autophagy (18,19). For example, while SLs were found to interact with growth-dependent signaling molecules in budding yeast (20), their cellular accumulation in monogenic yeast caused mitochondrial dysfunction and cell death (21). While early studies focused upon the role of entire SLs classes in cellular processes and
disease states, current evidences suggest that each molecule, i.e., ceramide (Cer) and sphingomyelin (SM), is functionally distinct and regulate wide varieties of biological functions (22-24). Various classes of glycosphingolipids accumulate in the midbodies and cleavage furrows of dividing mammalian cells (25). A recent report indicates that SLs are involved in maintaining a homeostatic balance of phosphoinositides (PIs) between trans-Golgi network and post-Golgi compartments, which is critical for Golgi-mediated cellular trafficking (26). A previous study also suggests that SLs regulate the release of exosomes that promote the clearance of amyloid-β fibril associated with Alzheimer’s disease (27).

*Giardia* is unique in the sense that only five SL metabolic genes have been annotated in the genome database (Figure 4) (28,29), and their functional identities are similar to eukaryotic SL enzymes (with low gene sequence identity). These five genes include two copies of giardial serine-palmitoyltransferases (i.e., gspt-1 and gspt-2), one copy of giardial glucosylceramide transferase gene (gglct1), and two giardial acid sphingomyelinase-like phosphodiesterase genes (gasmasepd B and gasmasepd 3b). A transcriptomic analysis revealed that all five genes are transcribed and expressed differentially during encystation and cyst production (Figure 5) (30,31). Studies have also indicated that while serine-palmitoyltransferase (gSPT) enzymes regulate ceramide (Cer) endocytosis in *Giardia*, glucosylceramide transferase (gGlCt1) synthesizes glucosylceramide (GlcCer), which regulates giardial cell cycle, membrane trafficking, and encystation (32).
Figure 4. **The reduced sphingolipid biosynthetic pathway in Giardia.** Of the 10 enzymes in the eukaryotic sphingolipid pathway, *Giardia* only has the three enzymes annotated in blue due to its compact genome (30). Figure adapted from Hernandez et al., (2008).

**Post-induction of encystation, hours (PIE, h)**

Figure 5. **gGlcT1 expression is induced upon encystation.** Immunoblot showing gGlcT1 expression during encystation. CWP2 expression was used as a comparison to confirm induction of the encystation process. Antibodies used were against gGlcT1, CWP2, and PDI-2, an ER marker, was used as a loading control (31). Figure adapted from Robles-Martinez et al., (2017).
1.3 gGlcT1 Catalyzes the Synthesis of GlcCer and GalCer

It was demonstrated that the overexpression and knockdown of the gGlcT1 enzyme in *Giardia* interferes with the biogenesis of encystation-specific vesicles (ESVs) and reduces the viability of cysts in culture (33). Interestingly, the normalization of gGlcT1 expression restores ESV biogenesis and cyst viability, further establishing the regulatory role of gGlcT1 in encystation and cyst production. More recently, it was demonstrated that gGlcT1 is a dual-substrate enzyme and catalyzes the synthesis of both glucosylceramide (GlcCer) and galactosylceramide (GalCer), not lactosylceramide (LacCer) (31). The chromatogram below is the result of an enzymatic assay with the fluorescent substrate NBD-ceramide being converted into both GlcCer and GalCer (Figure 6). The dual functionality of gGlcT1 was confirmed with bioinformatic analysis (31). The analysis revealed that gGlcT1 contained two glucosylceramide transferase (CGlcT) domains and one galactosylceramide transferase (CGalT) domain (Figure 7).

This is revelatory information because in higher eukaryotes and mammalian species, there is an independent enzyme in charge of catalyzing each reaction. In addition, these enzymes are localized to different organelle membranes with galactosylceramide transferase on the ER and glucosylceramide synthase on the Golgi apparatus (Figure 8) (34). *Giardia* not only has a compact genome, but it also lacks emblematic eukaryotic organelles such as the Golgi, mitochondria, lysosomes, etc. (28). This suggests that *Giardia* has adapted to a “minimalistic lifestyle” and uses one enzyme for generating multiple products (35). For this reason, the giardial GlcT1 enzyme is an advantageous target as it differs from the mammalian GlcT1, posing less risk for side effects from a gGlcT1-targeting treatment.
Figure 6. **gGlcT1 is a dual-substrate enzyme.** Live cells were labeled with the fluorescent substrate, NBD-Ceramide, to track product formation. Glycosphingolipids were extracted and ran on a thin-layer chromatography (TLC) silica-coated glass plate. The chromatogram displayed formation of both GlcCer and GalCer. It was thought that gGlcT1 could only catalyze the reaction from UDP-glucose to ceramide (to produce GlcCer), but the enzymatic assay demonstrated it can also transfer the galactose molecule from UDP-galactose to form GalCer (31). Figure adapted from Robles-Martinez et al., (2017).
Figure 7. **Bioinformatic analysis confirms gGlcT1 has multiple potential catalytic domains.** Bioinformatics predicted two glucosylceramide transferase domains and one galactosylceramide transferase domain (31). Figure adapted from Robles-Martinez et al., (2017).

Figure 8. **Mammalians have independently localized enzymes for synthesizing glucosylceramide and galactosylceramide.** Unlike *Giardia* that has one enzyme catalyzing
both reactions, higher eukaryotes and mammals have separate enzymes dedicated for each reaction making gGlcT1 distinct enough that it can be used as a potential drug target (31,34). Figure adapted from Ichikawa & Hirabayashi, (1998).

1.4 gGlcT1 and Lipid Homeostasis

It has been shown previously that gGlcT1 overexpression elevates the intracellular levels of lipids and fatty acids in trophozoites (32,33). Duarte et al. (2019) demonstrated that *Giardia* has dynamic sphingolipid metabolic pathways which alters during the transition from trophozoites to cysts (36). Ellis et al. recently found that lipid profiles in trophozoites and cysts are substantially different from each other (37). It further suggested that lipid metabolism in cysts is highly active and generates membrane lipids with long-chain fatty acyl moieties (37), suggesting that rapid membrane remodeling occurs during excystation. Since lipids are involved in membrane synthesis, it is likely that gGlcT1 plays a critical role in regulating lipid compositions of membranes (trophozoites and cysts). In another experiment, we found out that in-vitro cysts that were derived from different gGlcT1 expression cell lines (i.e., Endo-gGlcT1-HA, α2-Tub-gGlcT1-AU1, and OCT-gGlcT1-HA) (please see the methods section for additional information) displayed different levels of lipids in wild type and gGlcT1 over expression with two different constructs (Figures 11-12). These results (unpublished) indicate that the modulation of gGlcT1 activity affects lipid profiles in cyst and this could be associated with cyst viability and excystation by *Giardia*. This is interesting because *Giardia* has a limited lipid synthesis ability and mostly depends on supplies from outside (17,38,39). To further understand this phenomenon, we searched the genome database (GiardiaDB) and found that it has only one copy of long chain fatty acid elongase gene (gfaelo; GL50581_2228)
that could participate in lipid remodeling reactions induced by gGlcT1 (30). A sequence alignment of this gene found it had remarkable similarity to elongase genes of the thale cress plant Arabidopsis thaliana and another protozoan Entamoeba histolytica, but not the human gene (Figure 9).

Because long-chain fatty acids (LCFAs) participate in the remodeling of glycerol- and sphingosine-based phospholipids, membrane regeneration and dynamics (40), and cyst membranes undergo a dramatic reorganization becoming the plasma membrane of trophozoites (41), we cloned and overexpressed gFAELO in gGlcT1 expressing cells and monitored cyst formation to elucidate the interplay and regulation between these two enzymes. Labeling cysts with a cholera-toxin conjugate as a marker for the plasma membrane demonstrates how this membrane becomes the membrane of newly-excysted trophozoites or excyzoites (Figure 10) (41).

Figure 9. gFAELO sequence alignments shows similarity to A. thaliana and E. histolytica.

Comparing the only giardial fatty acid elongase reveals great similarity to multiple elongase genes of Arabidopsis thaliana and another protozoan, Entamoeba histolytica.
The cyst plasma membrane becomes the plasma membranes of excysted trophozoites. The excystation process of *Giardia* involves reorganization of the inner cyst membrane to give rise to two excyzoites (41).

**1.5 Current Treatments and Drug Resistance Phenomena**

As no vaccines are currently available to treat giardiasis, chemotherapeutic agents are the only options to control infection. The 5-nitroimidazole, metronidazole (Mtz,Flagyl®), is a commonly prescribed treatment for giardiasis. Although drugs such as tinidazole (5-nitroimidazole), nitazoxanide (nitrothiazole), albendazole (benzimidazole), and mebendazole (benzimidazole) are also used for treatment, they all may cause side effects (42). For example, Mtz is a genotoxic agent and a potential carcinogen, and its frequent use gives rise to drug-resistant giardiasis. Refractory or reoccurring giardiasis in endemic areas can lead to a drug resistance rate in up to 70% of cases (43). Other drugs from the nitrothiazole and benzimidazole groups are also used, but like Mtz, they can produce toxicity and may interfere with pregnancy (42). Among the non-metronidazole classes of compounds, several repurposed drugs such as auranofin, fumagillin (antibiotic), orlistat, omeprazole, disulfiram, and NBDHEX have showed promising activity against the *Giardia* parasite in culture (42). In addition to drug resistance and side effects,
poverty poses a barrier to treatment. This is especially problematic in developing countries where clean water is difficult to access (10). This public health issue makes it an urgent matter to find a new target for giardiasis treatment. I propose that gGlcT1 is unique and could serve as a novel target for developing anti-giardial agents in the future.

1.6 Specific Aims and Hypothesis

The overall goal of my research is to delineate the role of gGlcT1 in Giardia and the mechanism by which it regulates encystation and cyst production. I will be investigating: (1) whether both GlcCer and GalCer transferase activities are essential for inducing encystation; (2) if the regulated expression of gGlcT1 and gFAELO is critical for cyst morphology and viability. Therefore, this project aims to understand the function of gGlcT1 at the molecular level and its role in maintaining giardial virulence to pave the path for a more specific therapeutic target against giardiasis.

Specific Aim 1: Characterization of GlcT1 by molecular and biochemical methodologies. My preliminary results show that gGlcT1 is unique because: (1) it can catalyze the synthesis of glucosylceramide (GlcCer) and galactosylceramide (GalCer) and (2) consists of separate domains of ceramide-glucosyltransferase (CGlcT) and ceramide-galactosyltransferase (CGalT). I have also observed promoter-specific expression of CGlcT and CGalT, implicating that these two domains act differently and could regulate different sets of cellular functions. Site-directed mutagenesis assays will be carried out in attempts to uncover the active site residues important for its dual-substrate activity. These results will be important to determine the function of each domain and their effects on cyst production, viability, and infection.
Specific Aim 2: Investigate the modulation of cyst morphology by gGlcT1 and a possible interplay with long-chain fatty acid elongase (gFAELO). The impact of the GlcT1 enzyme in context of giardiasis will ultimately be determined by cyst virulence in mouse models. For the parasite to induce infection in mice, the cysts have to be viable in terms of having the ability to excyst in the organism and encyst once again before being excreted. Because the different gGlcT1 clones generated in Specific Aim 1 show differential enzymatic activities, my goal is to test their impact on cyst morphology.

Impact: The proposed study will provide an in-depth understanding of the gGlcT1 and the possibility to use it as a target of drug development.

I hypothesize that elucidating the function of glucosylceramide transferase will reveal the mechanisms of giardial differentiation and could be exploited to design novel drugs against this pathogen.
2. METHODS

2.1 Giardia Culture

*Giardia* trophozoites (strain WB, ATCC No. 30957) were cultured in TYI-S-33 medium supplemented with adult bovine serum (ABS, 5%) and bovine bile (0.5%) at 37°C. The antibiotic piperacillin (100 μg/ml) was added during routine culturing of the parasite (44). Antibiotics such as blasticidin (50 μg/ml), neomycin (150 μg/ml), and puromycin (50 μg/ml) were also used for culturing endogenous and episomally-tagged gGlcT1 cells. Trophozoites were harvested by scraping and centrifuged at 1,500 x g for 5 minutes at 4°C, followed by three phosphate-buffered saline (PBS) washes. In vitro encystation was carried out following the method described earlier (13). Briefly, trophozoites were cultured in TYI-S-33 medium with (pH 7.8) containing adult bovine serum (10%), lactic acid (10%), and porcine bile (5%) (13). Cells were allowed to encyst for 72 hours (h) and harvested by centrifugation at 1,000 x g for 5 minutes at 4°C, followed by subsequent sterile water washes to isolate water-resistant cysts.

2.2 Plasmid Construction

A plasmid containing an α-tubulin promoter with an AU1 epitope was obtained from Dr. Chin-Hung Sun, Taiwan (45). The plasmid pKS-3HA-BSR was used to incorporate the endogenous tag plasmid (46). The giardial Mad2 gene (a mitotic check point protein, GL50803_100955) was previously incorporated to insert Ascl and Fsel restriction sites. The entire open reading frame of *gglct1* (GL50803_11642) was amplified and cloned into the plasmid using the primers: 5’ CGCGAAGCTTGGCCGCGCAGCATGGACGGGTGACTCTCTCC 3’ and 5’
GCGCCTCGAGCGGCGCGCGTCGAGGGATTTTTTATTGGC 3’. The insert and plasmid were digested with Ascl and FseI and ligated together, replacing the Mad2 gene (Figure 11).

The plasmid pPAC-OCTp-ANX3-3xHA-C (ornithine-carbamoyl transferase) promoter plasmid was developed in a similar procedure (Figure 12) (47). The giardial ANX3 gene was digested from the plasmid using BamHI and NotI and was replaced with the open reading frame of gglct1 that was obtained by using the primers 5’ AAAAAGGATCCGACGGGTTGACTCTCTCCTT 3’ and 5’ AAAAA GCGGCCGC GCAGGGAGTTTTTTATT 3’, with the BamHI and NotI sites underlined respectively. gfaelo (GL50581_2228) was cloned in the same manner using the primers 5’ AAAAAAGGATCCGCTGGCTGGTCATGGCTTCA 3’ and 5’ AAAAAAGCGGCCGC GCATTAACCATTTCAGTGCAA 3’. The start codon was already in the plasmid and the base pairs in red had to be inserted to compensate for a frameshift. Plasmids were transformed into chemically competent DH5a E. coli cells and plated onto LB plates with 100 ug/ml ampicillin. Colonies were screened by PCR and sequenced at the UTEP DNA Sequencing Core facility. A maxiprep was then performed to ensure sufficient quantity of DNA to be electroporated. Successful endogenous HA tag clones were linearized with FseI prior to electroporation for homologous recombination (46).
Figure 11. **The constructs that were used to incorporate an endogenous HA tag into gGlcT1 and gFAELO, respectively.** The pKS constructs without a promoter were used to homologously recombine at the endogenous locus in Giardia to insert the hemagglutinin epitope tag (46).
Overexpression constructs used for gGlcT1 and gFAELO modulation. (A) OCT promoter constructs were used for overexpression of gGlcT1 and gFAELO, while the (B) α-tubulin promoter construct was used to coexpress gGlcT1 with gFAELO (45,47).

2.3 Generation of mutant cell lines

Instructions were followed according to the QuikChange II XL kit (Agilent) to truncate the gglct1 gene in the OCT promoter-3x HA tag plasmid (Figure 13A). Complimentary primers were designed to flank the region of deletion, with 15 bases of the gene sequence on both sides. Using the gglct1 sequence alignment as a guide, the deletions were chosen to be amino acids 54-80 (-CGlcT1), 148-179 (-CGlcT2), and 242-267 (-CGalT) as seen in Figure 13B (31). The primers for the deletions were the following: 5' GTCGTTCTGCCCTGCTGTACGTGTTGAG 3' and 5' CTCAACAACGTACACGCAAGGCAAGAACGAC 3'; 5' CTCGAAGCTCACAGAGCCTTGTGTTTGCC 3' and 5' GGCAAACACAAGGCTCTGTGAGCTTCGAG 3'; 5' GTCCCTTTTTGCTGGTATTCAAGCTCACACGAC 3' and 5' GTGTCCTCTCTGAATA
CCAGCAAAAAGGGAC 3'. Colonies were screened by PCR and sequenced at the UTEP DNA Sequencing Core facility. The primers used for sequencing or confirming the domain deletions were 5’ CAAGAATCGTCCAAGGGTTA 3’ (-CGlcT1), 5’ CATGCTAGAGACGTCTCCTC 3’ (-CGlcT2), and 5’ CCAGCTCATAATGGTCTACC 3’ (-CGalT). A maxiprep was done on each clone to gather sufficient DNA material for electroporation.

Figure 13. Domain deletion strategy and notation. (A) The QuikChange II XL kit was used with primers flanking each domain to generate OCT promoter constructs with mutant gGlcT1. (B) The deletion mutants are referred to as -CGlcT1, -CGlcT2, and -CGalT,
indicating removal of the glucosylceramide or galactosylceramide transferase domains.

2.4 Transfection of Giardia trophozoites

Approximately 1×10⁷ trophozoites were resuspended in 300 μl of complete TYI-S-33 medium (pH 7.1) with 40 μg of plasmid and inserted into 4-mm electroporation cuvettes (31). Trophozoites were 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 then allowed to recuperate in media overnight, followed by selection with blasticidin (100 μg/ml), puromycin (100 μg/ml), or puromycin and G418 (150 μg/ml) (Fisher & Sigma Aldrich, St. Louis, MO). Media was replaced every three to four days. Stable transfectant trophozoites overexpressing gGlcT1 were established within two weeks.

2.5 Immunoblot

Cell lysates were prepared by incubating in hypotonic buffer for 30 minutes (5 mM HEPES-pH7.4, 0.5 mM EDTA, 0.1% Triton X-100) on ice followed by adding lysis buffer (50 mM HEPES, pH7.4, 250 mM sucrose, 25 mM KCl and 5 mM MnCl₂, 10 uM E64, and a protease inhibitor cocktail). Samples were sonicated using a 1/8” probe for 3 minutes with a 37% amplitude and 3 sec/9sec pulse-rest and passed through a 27-G X 1 1/4 needle 20 times. Protein concentration of cell lysates were determined using the Bradford assay (BioRad) and 30-40 μg of protein were boiled with 2x Laemmli Buffer and β-mercaptoethanol (BME) at 90°C for 5 minutes. The samples were then applied onto a polyacrylamide gel (10%) for 2 hours at 100V and 300 mA followed by an immunoblot analysis for 35 minutes at 110 V and 300 mA. The PVDF membrane was
then incubated overnight in the cold room with 5% milk/TBST (25 mM Tris-pH 7.5, 500 mM NaCl, 0.1% Tween 20) with a primary antibody. The mouse HA antibody (SIGMA) was used to detect HA-tagged gGlcT1 (dilution 1:1,000), while tubulin was detected from a mouse tubulin antibody that was a gift from Dr. Giulio Francia (dilution 1:100). The next day, the blots were washed three times with TBST buffer and incubated in the secondary antibody (Thermo) for an hour at room temperature (dilution 1:2,000). After three TBST washes, blots were covered with ECL solution (100 mM Tris-pH 8.8, 2.5 mM luminol, 0.4 mM 4-IPBA, 2.6 mM H₂O₂) and exposed using the iBright machine (Invitrogen).

2.6 Assessing cyst viability via flow cytometry

Water resistant cysts were stained with 1 mg/ml propidium iodide (PI) in PBS (Acros Organics). Samples were run on the Beckman Coulter Gallios flow cytometer. Gates were adjusted using control samples of 4% hydrogen peroxide treated cysts stained with PI and untreated cyst samples.

2.7 Determination of gGlcT1 activity by live-cell labeling

Live-cell NBD-ceramide labeling assay was performed to measure the gGlcT1 activity (Figure 14) (31). Briefly, ~1x10⁷ trophozoites were harvested and incubated in PBS for an hour at 37°C, shaking. The cells were then spun down at 1,500 g for 5 minutes and resuspended and incubated in 1.5 ml of incomplete DMEM media and 5 ul of 0.5 mM NBD-ceramide/BSA (Invitrogen) for 3 hours at 37°C with slow shaking. Glycosphingolipid (GSL) extraction was performed on the cell pellet by adding 1.3 ml of
water, 1.3 ml of chloroform, and 1.3 ml of acetic acid/methanol (1:50 v/v) into cell pellets. The tubes were vortexed for 1 minute and spun at 3,000 x g for 10 minutes. The bottom organic layer was transferred to a new tube, and 1.3 ml of chloroform was added to the original sample for another round of trace lipid extraction. Again, the tubes were vortexed and centrifuged in order to pool the second organic layer with the first one. The samples were dried for 20 min. under N₂ and resuspended in 80 ul of chloroform/methanol (1:1 v/v). 20 ul of the sample was spotted onto a silica gel 60 thin-layer chromatography (TLC) plate along with various concentration of standard lipids such as NBD-ceramide, NBD-glucosylceramide (NBD-GlcCer), NBD-galactosylceramide (NBD-GalCer), and NBD-lactosylceramide (NBD-LacCer) (Invitrogen and Matreya). The mobile phase for the chromatography was propanol, ethyl acetate, ammonium hydroxide, and water 75:5:5:25 (v/v/v/v). The plate was placed into a chamber for 2 h at room temperature and then dried for 20 minutes, prior to capturing images with the help of the GelDoc imager (BioRad). Fluorescent chromatograms were analyzed using the ImageJ software.

Figure 14. Flow-chart of the live-cell labeling with NBD-Ceramide protocol. Trophozoite or encysting cells were collected and starved, before being labeled with the fluorescent substrate NBD-Ceramide. The glycolipids were extracted and ran on a silica plate before being imaged and quantified on imaging software.
2.8 Confocal Microscopy

Trophozoites, encysting cells, and cysts were harvested and washed. Cell pellets were fixed in 50 μl PBS mixed with 250 μl of 4% paraformaldehyde for 10 minutes (33). Following PBS washes, the samples were blocked with normal goat serum (5%) for 20 min at room temperature. This was replaced with cyst antibody (Santa Cruz sc-57744) at a dilution of 1:100 in 1% NGS and incubated overnight at 4°C. Cells were washed with PBS before incubating with secondary anti-mouse antibody for an hour at room temperature in the dark at a dilution of 1:400 (Alexa Fluor 568, Invitrogen). Following PBS washes, 50 μl of 1 μg/ml DAPI was added and incubated for 15 minutes. Cells were then resuspended in PBS and mounted with Vectashield Antifade mounting media. Samples were imaged and analyzed (n=30) using the LSM 700 laser scanning confocal microscope and software (Carl Zeiss).

2.9 Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software. Data sets were processed using one-way or two-way ANOVA with Tukey’s multiple comparisons. *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.
3. RESULTS

3.1 Specific Aim 1: Characterize gGlcT1 by molecular and biochemical methodologies

3.1.1 Modulation of gGlcT1 expression in Giardia

Previous work from our laboratory has demonstrated that gGlcT1 expression is elevated during encystation and its unregulated expression interfered with ESV biogenesis and cyst production (31,33). In our previous experiments, we used an overexpression gGlcT1 plasmid that contained a weak α2-tubulin promoter. This promoter was found to be active when the cells were subjected to stimulation for encystation. Using this plasmid, Robles-Martinez et al., demonstrated that Giardia preferably expresses CGalT activity rather CGlcT, indicating that promoter-specific expression of CGlcT and CGalT domains are possible (31). Since gGlcT1 synthesis is induced during encystation and overexpression (~3 fold) of gGlcT1 under α2-tubulin promoter decreased cyst production and viability (33), I examined whether the expression of this enzyme in trophozoites with the help of a strong promoter would have an impact on cyst production and cyst viability. Therefore, gGlcT1 was overproduced under a strong ornithine-carbamoyl transferase (OCT) promoter (48). The construct pPAC-OCTp-ANX3-3xHA-C was obtained from Dr. Svärd (University of Uppsala) (47). Side by side, I also tagged endogenous gGlcT1 by homologous recombination as described in the “Methods Section,” using a construct (pKS-3HA-BSR) obtained from Dr. Alex Paredez (University of Washington) (46). Conventionally, gGlcT1 levels increase during encystation, OCTp-gGlcT1-HA driven by the OCT promoter was expressed in trophozoites and encysting cells, but its expression was dramatically reduced in cysts (Figure 15).
Figure 15. **OCT promoter overexpression cells show a unique gGlcT1 protein profile during differentiation.** Immunoblot of cells under the OCT promoter display gGlcT1 expression in trophozoites and encysting cells, but significantly lower in cysts. On the contrary, cells carrying Endo-gGlcT1-HA showed the synthesis of gGlcT1 in a stage-specific manner. Troph, trophozoites; E24, Cells with post-induction of encystation for 24 hrs (24h PIE)
3.1.2 Dissecting the function \textit{gGlcT1} catalytic domains

To determine which domain was more critical for enzymatic function, each domain was deleted utilizing site-directed mutagenesis. \textbf{Figure 16} compares the changes seen in trophozoites, encysting cells, and cyst production of the 5 different clones expressing \textit{gGlcT1}. These 5 clones are the: (1) endogenous-tagged \textit{gGlcT1}, (2) OCT promoter overexpressing \textit{gGlcT1}, and the three truncation mutants under the OCT promoter (-CGlcT1, -CGlcT2, and -CGalT). Trophozoite growth was tracked for a total of 32 hours, with counts every 8 hours to account for doubling time. I observed that while trophozoites showed more or less similar growth patterns, parasites lacking either catalytic domain (-CGlcT1 or -CGalT) drastically reduced the production of cysts (\textbf{Figure 16A and 1C}). Interestingly, however, the deletion of these catalytic domains didn't interfere with the biogenesis of the encystation-specific vesicles (ESVs) as their counts among cell lines remained unchanged (\textbf{Figure 16B}).

The next step was to determine the viability of the cysts that were produced by \textit{Giardia} cells with deleted catalytic domains. For this, water-resistant cysts isolated from all the cell lines were stained with PI and subjected to flow cytometry analysis as described in the Methods Section. Incidentally, no significant difference in viabilities were observed in \textit{gGlcT1} mutant clones as shown in \textbf{Figure 17}. Although less cysts were produced with mutant cells, their viability was consistent indicating that they retained the ability to excyst.
Figure 16. Comparison of the trophozoite growth and production of ESVs and cysts for the various clones of *Giardia* expressing gGlcT1. (A) *Giardia* cell lines were cultured in TYI-

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**Figure 16. Comparison of the trophozoite growth and production of ESVs and cysts for the various clones of *Giardia* expressing gGlcT1. (A) *Giardia* cell lines were cultured in TYI-**
S-33 medium supplemented with bovine serum (5%) and bovine bile (0.5%). The growth was initiated with $1 \times 10^4$ cells, and the growth was monitored for 32 hours (~4 doubling times) by counting with the help of a hemocytometer. (B) *Giardia* trophozoites were encysted for 24 hours in encysting media. They were harvested after 24 hours and fixed with 4% PFA before they were stained with cyst antibody (SC-57744) and Alexa 568. The number of encystation-specific vesicles (ESVs) were counted per cell. There was no significant difference among cell lines. (C) $5 \times 10^6$ trophozoites were cultured in TYI-S-33 medium with pH 7.8 and 10% adult bovine serum, 10% lactic acid, and 5% porcine bile. Quantification of cyst production after encystation for 72 hours showed a significant difference in the cyst production of mutants without the CGlcT1 or CGalT domains.

Figure 17. **Comparison of the cyst viability of the gGlcT1 cell lines.** Water-resistant cysts were subjected to propidium iodide staining and ran in the flow cytometer after a brief incubation. Cyst viabilities did not change among gGlcT1-expressing cell lines and mutants. Data analysis was performed with a one-way ANOVA and Tukey’s multiple comparison test. ** $p<0.01$, *** $p<0.001$. 

![Graph showing cyst viability comparison](image)
3.1.3. Analysis of reaction products

For the analysis of reaction products, wild type, overexpressed, and mutant (Figure 18A) gGlcT1 trophozoites were subjected to encystation for 24 hr (24 PIE) followed by in vivo labeling with fluorescently conjugated NBD-ceramide (31). The product formation was maximum when overexpressed cells were used and both GlcCer and GalCer were produced (Figure 18B-C). Although GlcCer levels were higher compared to GalCer, Figure 18B-C indicates that the CGlcT1 and CGalT domains are active because deletion of these domains reduced the product formation as evidenced by the fluorescent chromatogram and the quantification of the bands. There was no overall difference in galactosylceramide production among cell lines, but it is important to note that this product formation is induced upon encystation (data not pictured). These results indicate that there is a coordinated expression of both CGlcT and CGalT domains that regulate the activity of gGlcT1 during the growth and encystation of Giardia.

![Diagram of enzyme domains and deletion mutants]
Figure 18. Enzyme activity is increased in the overexpression cell line but reduced in mutants -CGlcT1 and -CGalT. (A) Corresponding notation for each deletion mutant. (B) Thin-layer chromatography plate displaying the glycosphingolipids labeled with fluorescently labeled NBD-Ceramide. T, trophozoites; E24, 24 hr PIE. (C) Quantification of enzyme activity assayed by thin-layer chromatography. Data analysis was performed with a two-way ANOVA and Tukey’s multiple comparison test. *p<0.05, ** p<0.01, ***p<0.001.
3.2 Specific Aim 2: Investigate the modulation of cyst morphology by gGlcT1 and a possible interplay with long-chain fatty acid elongase (gFAELO)

3.2.1 Cyst wall thickness, area, and diameter is affected by gGlcT1 mutations

Cyst morphology is a determinant for the giardial cyst to survive the environment and excyst upon entering hosts (41). An intact and thick cyst wall is critical for preventing permeability of water, solutes, and other molecules to sustain the tonicity allowing the resistant cysts to withstand environmental conditions. It has already been shown that deleting either CGlcT1 or CGalT results in decreased cyst production. With this in mind, a confocal analysis was performed to determine the changes in cyst morphology, including the cyst wall thickness to determine whether specific gGlcT1 domains are important in the cyst composition. Water-resistant cysts were fixed and stained with CWP antibody to stain the cyst wall with conjugate Alexa fluor 568. The immunostaining of cysts can be seen in Figures 19A-E. Cyst measurements were taken from multiple frames and were documented. Cyst diameter and cyst area of cysts without the CGlcT2 domain remained similar to wild-type (Figure 19F-G). Overall, cysts without the CGlcT1 and CGalT domain resulted in smaller cysts with a thinner cyst wall (Figures 19F-H). This data shows that the CGlcT1 and CGalT domains are not only important for cyst production and enzymatic activity, but also cyst morphology.
Figure 19. Changes in Cyst Parameters from gGlcT1 deletion mutations. (A) Cyst confocal images of Endo-gGlcT1-HA, (B) OCTp-gGlcT1-HA, (C) -CGlcT1, (D) -CGlcT2, and (E) -CGalT cysts. (F-H) Cyst wall thickness, cyst area, and cyst diameter among the different expressions of gGlcT1 demonstrate that -CGlcT1 and -CGalT cysts are smaller with a thinner cyst wall. Data analysis was performed with a one-way ANOVA and Tukey’s multiple comparison test. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
3.2.2 Interplay between glycosphingolipid production and lipid remodeling

It has been reported that excess production of gGlcT1 increases lipid and fatty acid uptake by *Giardia* and that alters its overall lipid homeostasis (33). However, the mechanism of gGlcT1-induced changes of lipid homeostasis is not clear. Since the long-chain fatty acids (LCFAs) participate in the remodeling of glycerol- and sphingosine-based phospholipids and membrane regeneration and dynamics, we cloned and overexpressed giardia fatty acid elongase-1 gene (*gfaelo-1*, GL50581_92729) in *Giardia* (39). It was also expressed under an OCT promoter by itself and transfected into cells with α-TUB-gGlcT1-AU1.

Immunoblot analysis showed that it is expressed in cysts but becomes constitutively expressed with the co-expression of +gGlcT1 (*Figure 20A*). This interplay was seen in the trophozoite growth, as these cells grew slightly faster or had an increased doubling rate when compared to wild type endogenously tagged cells and overexpressing gFAELO cells (*Figure 20B*). The impact of gFAELO was also seen when cyst production was measured in these cell lines. Overexpressing gFAELO on its own resulted in the least amount of cyst production and this phenotype was only slightly rescued upon the coexpression of gGlcT1 (*Figure 20C*). Altogether this data indicates that overexpressing gFAELO impacts cyst production, and when it is overexpressed with gGlcT1, the interplay between both enzymes leads to overall metabolic changes giving rise to changes in both trophozoite growth and cyst production. The phenomenon of a distinct global profile was seen in a PCA of extensive lipidomic analysis in cysts (unpublished).
A

60 kDa →

+gFAELO +gGlcT1

50 kDa →

Tubulin

B

Trophozoite Growth Curves

Cell Count

1.5×10^6

1×10^6

5×10^5

0

0 10 20 30

Hours

Endogenous gFAELO
+gFAELO +gGlcT1 +gFAELO

C

Cyst Production

% Encystation

80

60

40

20

0

Endogenous gFAELO +gFAELO +gGlcT1 +gFAELO

Giardia Cell Line
Figure 20. **Coexpression of gFAELO and gGlcT1 alters gFAELO expression, trophozoite growth, and cyst production.** (A) Overexpressing gGlcT1 under the alpha-tubulin promoter (α-TUB-gGlcT1-AU1) and gFAELO under the OCT promoter (OCTp-gFAELO-HA) induces constitutive expression of gFAELO. (B) Trophozoite growth was higher in gFAELO and gGlcT1 co-expressing cells. (C) Cyst production was decreased when gFAELO was overexpressed alone or in conjunction with gGlcT1. Data analysis was performed with a one-way ANOVA and Tukey’s multiple comparison test. *p<0.05, ** p<0.01

To determine the effects on cyst morphology, water-resistant cysts were once again fixed and stained against the giardial CWP, bound to the conjugated Alexa fluor 568 (**Figure 21A-C**). It was found that cysts derived from gFAELO overexpressed cells are slightly larger with relatively thinner cyst walls, suggesting the gFAELO expression in cysts could be linked to excystation (**Figure 21D-F**). On the other hand, coexpression of gGlcT1 with gFAELO reverses the proves, further implicating that gFAELO and gGlcT1 are co-regulated and interact especially when it comes to cyst production and morphology. Confocal images and analysis demonstrated that overexpressing gFAELO with or without gGlcT1 influences cyst wall thickness, area, and diameter (**Figure 21**).
Figure 21. **Change of Cyst Parameters by gGlcT1 and gFAELO.** (A) Cyst confocal images of Endo-gFAELO-HA, (B) OCTp-FAELO-HA, and (C) α-TUB-gGlcT1-AU1 and OCTp-FAELO-HA cysts. (D-F) Cyst wall thickness, cyst area, and cyst diameter measured among different expression parameters of gGlcT1 and gFAELO. Data analysis was performed with a one-way ANOVA and Tukey’s multiple comparison test. ** p<0.01, **** p<0.0001.
4. DISCUSSION

Glucosylceramide transferase (GlcT1) is an evolutionarily conserved enzyme across eukaryotes and protozoan parasites. It is responsible for forming one of the most basic glycosphingolipid (GSL) building blocks, glucosylceramide (GlcCer) by combining UDP-sugar and ceramide as follows:

\[
\text{GlcT1} \\
\text{UDP-Glucose + Ceramide} \rightarrow \text{Glucosylceramide}
\]

The enzyme has been implicated for its role in cell proliferation and multi-drug resistance in cancers including but not limited to breast, neuroblastoma, melanoma, colon, and lung cancer (49-53). Inhibiting the expression of gGlcT1 makes cells more susceptible to chemotherapy and apoptosis (54-59). It has also been implicated in restoring insulin sensitivity and protective effects on renal and neuronal tissue (60-62). Therefore, it is being considered as a promising drug target in future cancer, renal hypertrophy, diabetes type 2, Parkinson’s, and Alzheimer’s treatments.

In \textit{Giardia}, gGlcT1 is an ER membrane protein (number of amino acids: 537; MW: \~55-60 kD) and sufficiently diverged from mammalian GlcT1. Pharmacological and molecular interference of gGlcT1 inhibits the attachment, growth, and encystation (30,32). Our two laboratories used a small molecule inhibitor called PPMP (threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) to inhibit the enzyme activity in \textit{Giardia}. PPMP is a known inhibitor of GlcT1 with therapeutic potential against cancer (63). Štefanić et al. demonstrated that PPMP inhibited the attachment, replication, and encystation of \textit{Giardia} in culture (32). Although it didn’t interfere with the cell cycle progression, they also found
that PPMP blocked cytokinesis in trophozoites. Our laboratory, on the other hand, demonstrated that PPMP inhibited the cyst production by blocking the expression of the \textit{gglct1} gene and also the nuclear division or karyokinesis of cysts (30). Furthermore, both Štefanić et al. and our laboratory demonstrated that gGlcT1 affects the lipid synthesis and remodeling in \textit{Giardia} trophozoites (32,33). However, the most fascinating aspect of gGlcT1 function was demonstrated by Mendez et al., who showed that the molecular modulation of gGlcT1 by overexpression and knockdown (using anti-gGlcT1 morpholino oligonucleotide) blocked ESV biogenesis and cyst production by \textit{Giardia} in culture (33).

Robles-Martinez et al. conducted a detailed analysis of this enzyme and found that gGlcT1 can catalyze the synthesis of two products (\textbf{Figure 6})—GlcCer and GalCer, but not mannosylceramide (ManCer) or lactosylceramide (LacCer) (31). This was an important observation as there currently is no galactosylceramide transferase (CGalT) enzyme annotated in the \textit{Giardia} genomic database. Therefore, my dissertation work aimed to further characterize the mechanism of this enzyme in its role of \textit{Giardia} differentiation and cyst production/viability. The objectives of my dissertation are: (1) to dissect the functions of the two catalytic domains on regulating the cyst formation and cyst wall biosynthesis, and (2) to investigate how gGlcT1 function is downregulated in cysts, which is critical for the successful excystation.

To fulfill the first objective, I employed a plasmid with a strong and natural promoter (OCT) containing gGlcT1 with hemagglutinin (HA) tag (\textbf{Figure 12A}) provided by Dr. Staffan Svärd (Uppsala). Using this newly assembled plasmid (OCT-gGlcT1-HA), it was possible to overexpress gGlcT1 in trophozoites as shown in \textbf{Figure 15}. Interestingly, it was observed that early expression of gGlcT1 in trophozoites prevents the synthesis of
this enzyme in cysts, in contrast to wild type, where gGlcT1 was mostly expressed in encysting cells and cysts (Figure 15). The same expression pattern of gGlcT1 in wild type was also observed with the plasmid (α2-tubulin-gGlcT1-AU1) that was used by Mendez et al. and Robles-Martinez et al. and shown in Figure 5 (31,33). Although the reason for this discrepancy of gGlcT1 expression with OCT-gGlcT1-HA is still not clear, it is possible that the early expression of this encystation-specific protein in non-encysting trophozoites could be an obstruction for the smooth transition of trophozoites to cysts, and therefore, is degraded by proteasomal degradation pathways (64). This observation can be further supported by the results of increased gGlcT1 activity in overexpressed cells (Figure 18), the reduction of cyst production (Figure 16) without affecting cyst viability (Figure 17), and the impact on cyst morphologies (Figure 19). Interestingly, cell lines lacking either of the catalytic domains revealed reduced enzymatic activities, decreased cyst production, and affected cyst parameters, specifically the thickness of the cyst walls, indicating both domains contribute significantly to cyst formation and cyst structures. Together they ultimately dictate the overall functions of the gGlcT1 enzyme (Figures 16-19).

The reduction of cyst formation and decreased cyst wall thickness by gGlcT1 mutant Giardia cell lines suggest that there should be a mechanism of controlling gGlcT1 expression that is critical for driving the process of excystation (Figures 16 and Figure 19). Since cysts are metabolically active and show a robust change of lipid profiles (Ellis et al., unpublished), we thought fatty acid elongase (gFAELO) could be involved in this event and responsible for the downregulation of gGlcT1 during excystation. Therefore, I expressed gFAELO and monitored its expression in trophozoites and encysting cells (Figure 12A). Figure 20A demonstrates that gFAELO is not expressed in trophozoites.
Its expression increases with encystation and is expressed predominantly in cysts. To further explore the idea if gGlcT1 and gFAELO interact, I co-expressed these two genes and monitored by western blot analysis (Figure 12). Results demonstrated that the co-expression changed the profile of gFAELO expression and instead of cysts, its synthesis was observed in all stages (Figure 20A). As far as cyst production is concerned, gFAELO reduced the production of cysts and generated cysts with thinner cyst walls (Figure 20C and Figure 21). This observation is in good agreement with our hypothesis that gFAELO might be associated with the excystation process. Co-expression of gFAELO with gGlcT1 slightly improves the effect of gGlcT1 (i.e., the formation of cysts with thick cyst walls in Figure 21). These results indicate that gGlcT1 and gFAELO may have opposite effects of each other. While gGlcT1 facilitates the cysts formation, gFAELO participates in excystation and the interplay between these two enzymes are critical for running the encystation-excystation cycle of Giardia.

4.1 Final Overview

Taking all the data into consideration, it is evident that gGlcT1 and gFAELO have important roles in the life cycle of Giardia. It was found that gGlcT1 affects giardial differentiation and cyst morphology, leading to implications in the ability for the parasite to withstand environmental conditions for transmission. It was also observed that the enzyme may have domain-specific enzymatic activity, and its interaction with the gFAELO enzyme involved in lipid remodeling may lead to a role in excystation as well. In this study, the implication of glucosylceramide synthase/transferase was further elucidated in the context of encystation and overall cellular function in Giardia.
From deleting domains in gGlcT1 and modulating gFAELO expression, it was found that although cysts resist water and their viability remained the same, they are ready and able to excyst and infect (Figures 19 and 21). Since cellular conditions are being changed, it was hypothesized that this would cause perturbations to the parasite’s livelihood. gGlcT1 is induced upon encystation and it needs either its first or third catalytic domain in order to produce its products glucosylceramide and galactosylceramide. Modulating expression or eliminating these domains has been shown to decrease cyst production and altered cyst morphology. In the same manner, overexpressing gFAELO lead to similar results but with greater importance in excystation. It is the enzymes’ impact on cyst morphology that will impact the parasite’s future ability to infect and be transmitted.

It can be postulated that modulation of gGlcT1 and gFAELO causes global changes in the encystation, infection, and transmission of Giardia (Figure 22). Future work will focus on finding the precise catalytic regions of the enzyme as to investigate the ultimate relevance of gGlcT1-related molecular changes on parasite infectivity and host-parasite interactions. Lipidomic analysis will also be performed to assess the global changes in the lipid profiles to determine how the two enzymes are interacting at the molecular level. This will confirm or deny that proper lipid homeostasis is important for both encystation and excystation.
gGlcT1 is known to be induced upon encystation, but without the CGlCt1 or CGalT domains, the cysts are smaller with thinner cyst walls most likely resulting in permeable cysts unable to withstand environmental conditions. gFAELO is also induced upon encystation and persists through the cyst. Due to its role in global lipid remodeling and a thinner cyst wall, it is predicted to have a role in facilitating the excystation process where the cyst plasma membrane is reorganized to become the plasma membrane of the two trophozoites that emerge and colonize a host’s small intestine.
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CURRICULUM VITA

Vanessa Enriquez was born in Cuidad Juarez, Mexico and was raised in Perryton, Texas. She later returned to live in El Paso, Texas. Her inquisitive mind blossomed in her high school freshman year biology course, which was the deciding moment for her to pursue biology for her undergraduate studies. Her initial goal was to enroll in medical school upon completion of her undergraduate degree, but she felt at home in the learning environment of infectious disease research in Dr. Das’ lab. Herein began her journey of studying the molecular mechanisms of Giardia lamblia.

She obtained her Bachelor of Science in the spring of 2016 from the University of Texas at El Paso. She was awarded both undergraduate and graduate RISE fellowships to continue her studies in molecular biology. She started the PhD program in the department of biological sciences in the fall of 2016. For the first year of her graduate career, she was an assistant instructor where she taught 2 sections of introductory biology labs culminating in the education of at least 40 students per semester. She also participated in mentoring of fellow undergraduate students in the Das lab. She presented her work and received travel award funding for the Molecular Parasitology Meeting in 2019, the American Society of Cell Biology and Capitol Hill Day in 2019 and 2020, as well as the American Society for Biochemistry and the Rio Grande American Society of Microbiology meetings in 2020. She hopes to build a career in clinical trials or the drug development process for diseases.

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