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Characterization of DNA Methylation in *Giardia intestinalis*

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Abstract

G. intestinalis is an intestinal protozoan parasite that causes 180 million symptomatic diarrhea cases (giardiasis) and more than 0.5 billion asymptomatic infections per year. Symptomatic infections are usually treated with metronidazole (Flagyl). However, resistance is emerging, and an alternative treatment is required. The mechanism to which the parasite causes the disease is not understood and, neither is the regulation of encystation (a process where trophozoites differentiate to cyst) However, preliminary data suggest that an epigenetic mechanism is involved. DNA methylation is an important epigenetic regulator in many organisms, but it is not known if the DNA is methylated in *Giardia.* The main goal of this project was to characterize DNA methylation in *G. intestinalis* and, if it exists, study if it is linked to cell differentiation and use it as a target for drug treatment. We found out using the dot blot technique complemented with immunofluorescence assays, that 6mA and 5mC DNA methylation exists on the genomic DNA of the assemblage A *G. intestinalis* isolate WB. 6mA methylation was also found on RNA. However, no major differences were detected between trophozoites and cysts. Assemblage B *Giardia* isolates (GS and H3) also have methylated genomic DNA, but we detected lower levels of methylation. A bioinformatic search was performed in the *G. intestinalis* WB genome in an attempt to identify DNA methylases. Expression levels through-out the life cycle, sequence similarities and structural modelling using iTASSER identified six putative DNA methylases in the WB genome. The six DNA methylases were over-expressed in *Giardia*, three were lethal and three localized to the nucleus. 5-azacytidine and 5-aza-2'-deoxycytidine nucleoside analog drugs prevent methylation and are incorporated into RNA and DNA, respectively. We tested these two drugs on *Giardia* trophozoites, and both have effects on the trophozoite stage (IC_{50} 1.46±0.46 μM for 5-aza-2'-deoxycytidine and 111±24 μM for 5-Azacytidine). The 5-aza-2'-deoxycytidine drug is actually more effective than metronidazole, showing that nucleoside analogs affecting DNA methylation could be alternative drugs for treatment of giardiasis.

List of abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
E2F	Elongation factor 2
gDNA	Genomic DNA
NF kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
pH	Power of hydrogen
6mA	N6-methyladenosine

5mC	5-methylcytosine
FTO	Obesity associated factor
IFA	Immunofluorescence assay
DAPI	4′,6-diamidino-2-phenylindole
PCR	Polymerase chain reaction
PDB	Protein data bank
tRNA	transfer ribonucleic acid
rRNA	ribosomal ribonucleic acid
5-aza-CTP	5-azacytidinetriosphosphate
5-aza-dCTP	5-azadeoxytriosphosphate
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris acetate EDTA
DNMT	DNA methyltransferase
CpG	Cytosine-phosphate-Guanine
RE	Restriction enzyme
FW	Forward
RV	Reverse
DIC	Differential interference contrast
alkB	Alpha-ketoglutarate-dependent dioxygenase
SAM	S-adenosyl-L-methionine
SAH	S-Adenosyl homocysteine
GCD	Glucocerebrosidase

1. Introduction

Giardia intestinalis is an anaerobic unicellular eukaryotic organism classified under metamonad in a phylogenic tree[1], a bi-nucleated flagellated protozoan parasite of the order Diplomonadida[2]. The parasite is classified into assemblages from A to H based on their genetically unique housekeeping genes such as glutamate dehydrogenase and, triose phosphate isomerase[3]. Assemblages are also distinct from each other in pathogenicity and clinical presentation[4]. Out of these assemblages, A and B are found to infect humans and animals[5]. The parasite has two developmental stages; infectious cyst (metabolically dormant) and the disease causing trophozoite stage[6]. Once the cyst is ingested through contaminated water or food, the cyst starts to differentiate into trophozoites in a process known as excystation[6]. Differentiation starts as the cyst travels through the stomach due to acidic pH and proteases that are present in the stomach, an amount of 10 cysts are capable of establishing an infection[7,8]. The trophozoites attach to the walls of the intestinal epithelial cells with their adhesive disc, which is said to be their main virulence factor[6]. Attachment with the adhesive disc prevents peristalsis, and thus the parasites are able to multiply in the host[7]. Trophozoites are transformed back to a cyst in a long process called encystation[6]. A high level of bile, and low pH triggers the process as the trophozoites passed down to the colon[9]. These conditions in the colon are not favorable for the trophozoites so, they start to differentiate by producing a resistant protein wall called cyst wall proteins, disassembling their adhesive disc, and lose their flagella to be later excreted out into the environment through feces[9]. When contaminated water or food is ingested, the cyst establishes the infection and the trophozoites cause the disease, and the cycle starts again[10].

Giardia intestinalis and other protozoan parasites such as *Plasmodium falciparum, Cryptosporidium* are collectively affecting the entire world and are major contributors to morbidity and mortality. Beyond the human health consequences, these eukaryotic pathogens are also socially and economically costly and constitute serious constraints on economic development[6]. Thus, successful elimination and eradication of diseases caused by protozoa and helminths would have an inconceivable impact on public health and quality of life. Unfortunately, for the majority of parasites, vaccines are non-existing, drug availability is scarce and drugs of current therapeutic portfolios suffer from rapid development of resistance[9].

Giardia causes the disease called giardiasis, with symptoms of diarrhea, weight loss, nausea[10]. *G. intestinalis* causes 180 million symptomatic diarrhea cases and more than 0.5 billion asymptomatic infections per year[6]. Symptomatic infections are usually treated with metronidazole (Flagyl) but resistance is emerging slowly[9] and there is a need to develop alternative treatments. One alternative target for treatment is epigenetic modifications and here we will investigate if the methylation machinery is a target for new drugs.

Epigenetics is described as heritable change in gene expression that occur without changes in DNA sequence[11]. Epigenetic modifications can regulate gene expression with different mechanisms such as interfering with transcription initiation by preventing binding of specific cellular factors, e.g. NF-kB, E2F and the RNA polymerase itself in human cells[12,13]. Epigenetic changes also alter the overall chromatin structure, indirectly affecting gene accessibility[12]. Unicellular yeast uses its yeast prion to epigenetically regulate its survival in adverse conditions[14]. Eukaryotes uses this mechanisms to regulate the signaling activity of a gene[15] and gene silencing at the chromatin level in eukaryotic organism is important for regulating key biological processes such as differentiation, imprinting and silencing of the X chromosome[16]. There are several forms of epigenetic modifications which include different types of histone modifications[17] but also DNA and RNA modifications. RNA molecules, most of all stable RNAs, display a large number of modifications of the bases and sugars[18]. DNA methylation was first identified in bacteria in 1953 in a phage infection, where the bacteria uses this modification to differentiate between it's own DNA from extraneous DNA of the phage[15]. 5-methylcytosine (5mC) has been widely studied and is said to be the most abundant modification in eukaryotes and tend to function to regulate gene expression[19]. DNA methylation on cytosine occurs at both CpG and non-CpG regions[20,21]. Human DNA methyltransferase 3 (Dnmt3) has been reported to catalyze 5mC methylation at CpG regions with higher frequency than at non-CpG regions in embryonic stem cells[21]. CpG dinucleotide is known to be the preferred site for methylation but methylation has been found in non CpG sites of the nf1 gene in early embryonic mouse development[20]. The 6-methyladenine (6mA) modification of DNA was initially found in bacteria but it has also been found in archaea and fungi[22]. 6mA modification in prokaryotes has been proven to regulate DNA replication, mismatch repair, transposition, transcription and host defense [23]. This is the dominant DNA modification in bacteria, but it is often only present in low amounts or not at all in the DNA of eukaryotes[24]. However, this modification has been found in eukaryotic mRNA and plays a crucial role in mRNA metabolism[25].

Recently the 6mA DNA modification has also been reported to exist in low levels in eukaryotes like *C. elegans*[26], Drosophila[22], *Trichomonas vigalis*[27] and Chlamydomonas[28]. In prokaryotes, mettl4 methyltransferase has adenine methyltransferase activity[29]. DNA N6 adenine methyltransferase 1 (DAMT-1) in eukaryotic cells has been reported to possess methyltransferase activity. This has been proven by a decrease in 6mA levels when DAMT-1 is depleted and an increase in 6mA levels when DAMT-1 is overexpressed in *C. elegans*[26]. Analyses indicates that DAMT-1 has a MT70 domain, which is common in eukaryotes[30]. Formation of 6mA is a reversible process[31]. 6mA "writers" with methyltransferase activity consist of three individual proteins; METL3, MTTL14 and William's tumor-associated protein WTAP[32]. "Erasers" are demethylases, which includes obesity associated (FTO) and alkB homolog5, "readers" are enzymes that detect the state of methylation on the DNA or RNA[31,33] and determine what happens to the RNA transcript; splicing and transport of the transcript, stability or translation of the transcript[25].

DNA methylation plays a crucial role in mediating epigenetics silencing of cancer-related genes, anti-cancer drug works by halting methylation through inhibition of the enzyme (Dnmt1) that methylates the DNA[34]. Out of many anti-cancer therapies[35], here we focus on 5-azacytidine and 5-aza-2'deoxycytidine, pyrimidine nucleoside analogs which were first discovered to be highly effective cancer static agent[36]. The former incorporated 80-90% into RNA, 10-20% into DNA[34], and the latter incorporate into DNA and inhibit the activity of Dnmt[37]. They both are pro-drugs and after a series of phosphorylation events during intracellular metabolism by kinases they are converted into 5-azacytidinetriosphosphate (5-CTP) and 5-azadeoxytriosphosphate(5-dCTP), which are incorporated into RNA and DNA, respectively[37]. These covalently bind to Dnmt1 when it attempts to methylate a strand, resulting in deactivation of Dnmt1 and ultimately hypomethylation of the DNA[35]. Functional consequences of 5-azacytidine include alteration of translation initiation[38] while 5-aza-2'-deoxycytidine result in hypomethylation, DNA damage and inhibition of cell proliferation[37 34].

1.1 Aims of the study

The aims of this project were to

- determine if DNA methylation exists in *Giardia* and if so, what type it is?
- study DNA methylation in the genome of different strains of *Giardia intestinalis*
- identify and localize putative DNA methylases in *Giardia intestinalis*

- investigate if DNA methylation changes during cell differentiation
- screen drugs that might affect DNA methylation in *Giardia intestinalis*

2. Materials and methods

Cell culture: Reagents were obtained from Sigma Chemical co. *G. intestinalis* assemblage A isolate WB-C6 (ATTCC catalog number 50803) and isolate NF, assemblage B isolate GS (ATCC number 50581) and the H3 isolate were grown in TYI-S33 media supplemented with 10% heat inactivated adult bovine serum (Gibco, Thermo Fisher Scientific) and bovine bile of final concentration of 0.125 mg/mL at 37°C until confluency of about 70-80% is reached, and they are used for the experiments[32]. Cells were harvested by centrifugation at 800×g for 5 mins at 4 °C and washed three times with PBS.

DNA extraction: Proteinase K (Thermo Scientific, EO0491) was added to the harvested cells, lysis buffer containing 50 mM EDTA, 1% SDS, and 1 M DTT was added to lyse the cells. DNA was extracted using phenol: chloroform: isoamylalcohol with pH 8.0 (Sigma Life Science, 1003077574) and precipitated with one tenth volume sodium acetate (CH_3COONa) and two volumes of 100% ethanol. The DNA was further treated with RNase A (Thermo Scientific EN0531) and purified with Qiagen Genomic tip (20/G) according to the manufacturer's indications.

RNA extraction: TRizol reagent (Ambion, Life Technologies 15596018) was added to the harvested cells and incubated for five minutes at room temperature, chloroform was added and centrifuge at 12,000×g for 15 minutes at 4°C, the solution was precipitated with isopropanol at room temperature, washed with 75% ethanol and resuspended in sterile water. The extracted RNA was DNase treated using Turbo DNase I (Thermo Scientific EN0521) and incubated for 30 minutes at 37ºC. RNA was further isolated using phenol: chloroform: isoamylalcohol of pH 5.4 and precipitated with sodium acetate (one tenth volume) and 100% ethanol. The sample was washed with 70% ethanol and resuspended in sterile water.

Genotyping: the beta-giardin gene was PCR amplified using the forward primer G7 (5'-AAGCCCGACGACCTCACCCGCAGTGC-3') and the reverse (5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3') as described in *Caccio et al*,[4] from the extracted genomic DNA of the different strains. The amplified genes were sent for sequencing (sanger

sequencing) using the primers used earlier for amplification at Eurofins genomics. The sequencing results were blasted on the NCBI website to confirm the identity of the strains used for the experiments.

Dot blot technique: Concentrated DNA (200 ng) of each sample was spotted on a nitrocellulose filter, crossed linked twice at 700 µj/cm^2, the membrane was blocked with 5% milk, 5% BSA dissolved in 0.1% tween 20 (Sigma Life Science, P9416) in tris buffer saline (TBS-T) for one hour at room temperature. The membrane was incubated with a primary antibody of concentration 1:1000 (polyclonal N6-methyladenosine antibody 6mA from Synaptic systems, monoclonal 5-methylcytosine antibody 5mC from Abcam) overnight at 4°C. The membrane was washed four times with TBS-T, and HRP conjugated secondary antibody of concentration 1:10000 was added, then incubated for two hours at room temperature. The signal was developed using the Bio-Rad clarity western ECL substrate, for 5 minutes and visualized in a GelDoc.

Immunofluorescent analysis of detecting methylation: Methylation was detected in different strains of *Giardia* trophozoites and WB strain cysts using antibodies that target 5mC and 6mA. Trophozoites and cysts were prepared as described above and placed on pre warmed microscopic slides. The cells were allowed to attach before fixation with 4% paraformaldehyde for 30 minutes at 37 °C. Water resistant cysts were air dried and fixed with 2% PFA. Fixation was followed by permeabilization with 0.1% Triton X-100. RNase A (100 µg, EN0531) was added and incubated for two hours and blocking with 5% BSA, 0.05%Triton X-100 at 4°C overnight. Primary antibody detecting 5mC and 6mA methylation (5-methylCytosine mouse monoclonal from Abcam 10805 /N6-methylAdenosine and polyclonal rabbit from Synaptic Systems) was added and incubated for 1 hour at room temperature. After washing the wells with blocking solution, fluorescently labelled secondary donkey anti-mouse Txred (Alexa Fluor Plus 594) and donkey anti-rabbit FITC (Alexa Fluor Plus 488) were added 5-methylcytosine and N6-methylAdenosine respectively in the dilution of of 1:2500 and incubated for 1 hour at room temperature in the dark. After washing was done six times with blocking solution the slides were mounted Vectasheild containing DAPI (Cat. no. H-200, Vector Laboratories), the slides were viewed in Nikon Eclipse Ti fluorescence microscope and images were processed with NIS-element AR software version 4.50.00.

Selection of Putative Methyltransferases: Giardia DB (www.giardiadb.org) was used to compile fifty-nine putative methyltransferases in *Giardia* with their annotations and *in vivo* expression levels. Six out of the fifty-nine of these were selected based on their structural similarities to known

DNA methylases as model on I-TTASER (protein structure and function prediction server). Also their conservation between assemblage A and B.

Cloning: Primers for the selected enzymes were designed for cloning into a pPAC-X3HA-MCs plasmid vector containing ampicillin and puromycin resistance genes using Benchling [Biology software]. (2020). Each gene including the promotor region was amplified from the WB strain of *Giardia intestinalis* with Phusion II DNA polymerase and dNTP from Thermo Scientific. The amplified genes were digested using FastDigest restriction enzymes (Thermo Scientific, FD0594, FD0504, FD0573) (Table 1) and were purified using Thermo Scientific GeneJET Gel extraction kit according to the manufacturer's indication. The digested samples were ligated with the pPAC-X3HA-MCs plasmid vector using T4 DNA ligase (Thermo Scientific, EL0011). The ligation mixture was transformed into DH5α competent cells.

Plasmid isolation: The plasmid was purified using Plasmid Isolation kit Column (K0692, Thermo Scientific). A single bacterial colony with the appropriate plasmid was inoculated in 50 mL lysogeny broth media and incubated on a shaker overnight at 37°C. Cells were centrifuged at 3000×*g* for 10 minutes at 4°C, and resuspended in resuspension buffer, adding 50 µg/ml RNase A, lysis, and neutralization buffer and after centrifugation at 13,200×*g* for 10 mins at room temperature was performed. An equal volume of 96% ethanol was added and loaded on the column. The columns were washed using wash buffer, and the plasmid was eluted from the column using water.

Transfection: *Giardia* WB cells were transfected by electroporation of 20-40 µg of plasmids using a Gene Pulser instrument (Bio-Rad) with the following settings: 350V, 800Ω, 960µF for 80-98ms, 50 µg/mL of Puromycin was used for selection.

Localization of methyltransferase: Immunofluorescence assay was used to localize the enzyme in the transfectants. Transfectants trophozoites were grown to a confluency of 70-80% and harvested. They were centrifugated at 800×*g* for 5 minutes at 4°C, washed with PBS, and placed on a prewarmed microscope slide (Thermo Scientific, Cat. No. 637573). Trophozoites were induced to encyst for 22 hours and then harvested. They were washed with PBS once and placed on a pre-warmed microscopic slide and allowed to attach before fixation with 4% paraformaldehyde for 30 minutes at 37°C. Water resistant cysts were air dried and fixed with 2% PFA, followed by permeabilization with 0.1% Triton X-100 and blocking with 5% BSA 0.05% Triton X-100 at 4°C overnight. Fluorescently labelled primary monoclonal antibody

(Thermofisher Scientific, Alexa Fluor 594, Cat. No. A-21288) that detects hemagglutinin (HA) tag in dilution of 1:200 in blocking solution (0.05% Triton-X 100 and 2% BSA) was added into the wells and incubated for 1 hour at room temperature, after washing the wells six times with blocking solution, the slides were mounted with Vectashield containing DAPI (Cat. no. H-200, Vector Laboratories), the slides were visualized in Nikon Eclipse Ti fluorescence microscope and the images were processed with NIS-element AR software version 4.50.00.

Drug assay: To determine the effect of methylation targeting drugs 5-aza-2'-deoxycytidine (A3656, sigma) and 5-azacytidine on *Giardia*, the concentration of drug required to inhibit the cells by 50% (IC50) assay was carried out. WB cells were grown and chilled on ice for 15 minutes and detached by hitting, cells were enumerated in Neubauer chamber and diluted to 2×10^3 cells/mL, diluted cells were seeded in opaque 96-well plate, the plate was placed in an anaerobic sachet (AN0020D, Thermo Scientific) with an anaerobic pouch (AG0060, Thermo Scientific) and incubated at 37°C for two hours. Drug was added (100 µM, 50 µM, 20 µM, 10 µM, 5 µM, 2 µM, 1 µM) and incubated at 37°C for 72 hours, cell titer-Glo luminescent cell viability (Promega G7571) was used to read the plates according to the manufacturer's indications, and luminescence was measured with a Tecan (Infinite M 200 pro).

Table 1. List of designed primers and restriction enzymes used for amplification of the various genes

ORF	primer sequence FW 5' -3'	primer sequence RV 5' -3'	RE. FW	RE. RV
GL50803_1902	TCAAGCTTTGGTCATTGTGTCA CGGTAGCAA	TCGCGGCCGCGCGGCGCA TACATCTGTCTTTTGCC	Hind III	Not I
GL50803_1903	TCAAGCTTGACTCACTGGATGCACTCTCTG	TCGCGGCCGCGCTGGTAGTTTTTTGGAACAGAGC	Hind III	Not I
GL50803_8961	GCCCATGGATAGAACTGCCAAGTACCGTAGAAA	TCGCGGCCGCGCACCAAAGTAATTAGCAAAGAGCAG	Nco I	Not I
GL50803_21512	GCCCATGGTGGTCTCAGACGTAAGGCCAATGG	TCGCGGCCGCGCGAATAGGCTATGACCCCGTCTTCG	Nco I	Not I
GL50803_100887	TCAAGCTTAGGGACGTCCTCTGTAACG	TCGCGGCCGCGCCATCTTCAGCTTTCTGCGC	Hind III	Not I
GL50803_100959	GCCCATGGAAACCTGTCTGTATGACAACCGAA	TCGCGGCCGCGCGATATGATCACCTGTAAATTCCAATGC	Nco I	Not I

Table 2 specifications used for amplification of genes *(ORF)* using PCR

ORF	Cycles steps	Temperature (°C)	Time (s)	Cycles
GL50803_1902	Initial denaturing	98	30	1
	Denaturing	98	10	35
	Annealing	67.4	30	
	Extension	72	30	1
	Final extension	72	600	
GL50803_1903 GL50803_100887	Initial denaturing	98	30	1
	Denaturing	98	10	35
	Annealing	63.6	30	
	Extension	72	60	
	Final extension	72	600	1
GL50803_100959 GL50803_21512 GL50803_8691	Initial denaturing	98	30	1
	Denaturing	98	10	35
	Annealing	67.2	30	
	Extension	72	45	
	Final extension	72	600	1

3. Results

3.1 Identification of 6mA and 5mC in the genomic DNA of *G. intestinalis*.

In order to study DNA methylation in *Giardia* we used dot-blots to detect 6mA and 5mC modifications in different strains of *G.intestinalis* from both assemblages A and B. The NF and WB strains, which are both from assemblage A, and the GS and H3 strains, which are both assemblage B isolates, were used in the study. We first extracted genomic DNA (gDNA) from NF, WB, GS and H3 trophozoites grown *in vitro* (Figure 1A). Antibodies specific for the 5mC and 6mA modifications were used in dot-blots to assess the presence of the modifications. Before the experiment, we tested that the antibodies specifically recognized the two modifications using DNA from mouse cells (low or no 6mA-high 5mC) and *Drosophila* DNA (low 6mA and no 5mC) (Sup. File 1). The 5mC and 6mA DNA methylations were detected in gDNA from trophozoites of both assemblage A and B, but the strongest signal was obtained from the assemblage A strains (Figure 2A and B). The antibodies also recognize RNA modified by 5mC and 6mA but when extracted RNA from the WB trophozoites (Figure 1B) was tested we did not get any signal (Figure 2). DNA was also extracted from WB cysts and the level of DNA methylation was compared to DNA from trophozoites. This showed that both trophozoites and cysts contain methylated DNA (Figure 2C and D) but there were no major differences in intensity. However, when a time course was done during encystation variable levels were detected (Sup. File 1).

We also used immunofluorescence microscopy (IFL) to investigate the presence and localization of 5mC and 6mA in *Giardia* WB trophozoites and cysts (Figures. 3 to 6). The 5mC signal co-localize with the nuclei (DAPI-stained) in the trophozoites with and without RNase treatment (Figure 3A and B). The signal appears to be more focused in the RNase-treated cells (Figure 3B). Cysts are also stained in the four nuclei with and without RNAse treatment (Figure 4A and B). Many cysts are not stained, either reflecting lower levels or problems with penetration of antibodies into the cysts (Figure 4). Staining of trophozoites and cysts with the 6mA antibody (Figures. 5 and 6) suggests that there are 6mA modifications in both DNA and RNA in the WB strain since there are signals emanating from the cytoplasm when the cells are not treated with RNase (Figure 5A) and a co-localization with the nucleus when treated with RNase (Figure 5B). 6mA detection in cysts was not convincing since most of the antibody seem to have gotten stuck in the cyst wall (Figure 6). To conclude this section, our data suggest that there are 5mC and 6mA

DNA modifications in *Giardia* DNA. In contrast to other eukaryotes, unreported data suggest that, the level of 6mA might be higher than 5mC. Parasites from assemblage A have more modified DNA than assemblage B isolates.The modifications are found both in the trophozoite and cyst stages but changes in levels occur during encystation. We did not detect any RNA modified with 5mC but for 6mA we have conflicting results: dot blots show no signal whereas IFL with and without RNase treatment suggests that there is 6mA modified RNA in *Giardia*.

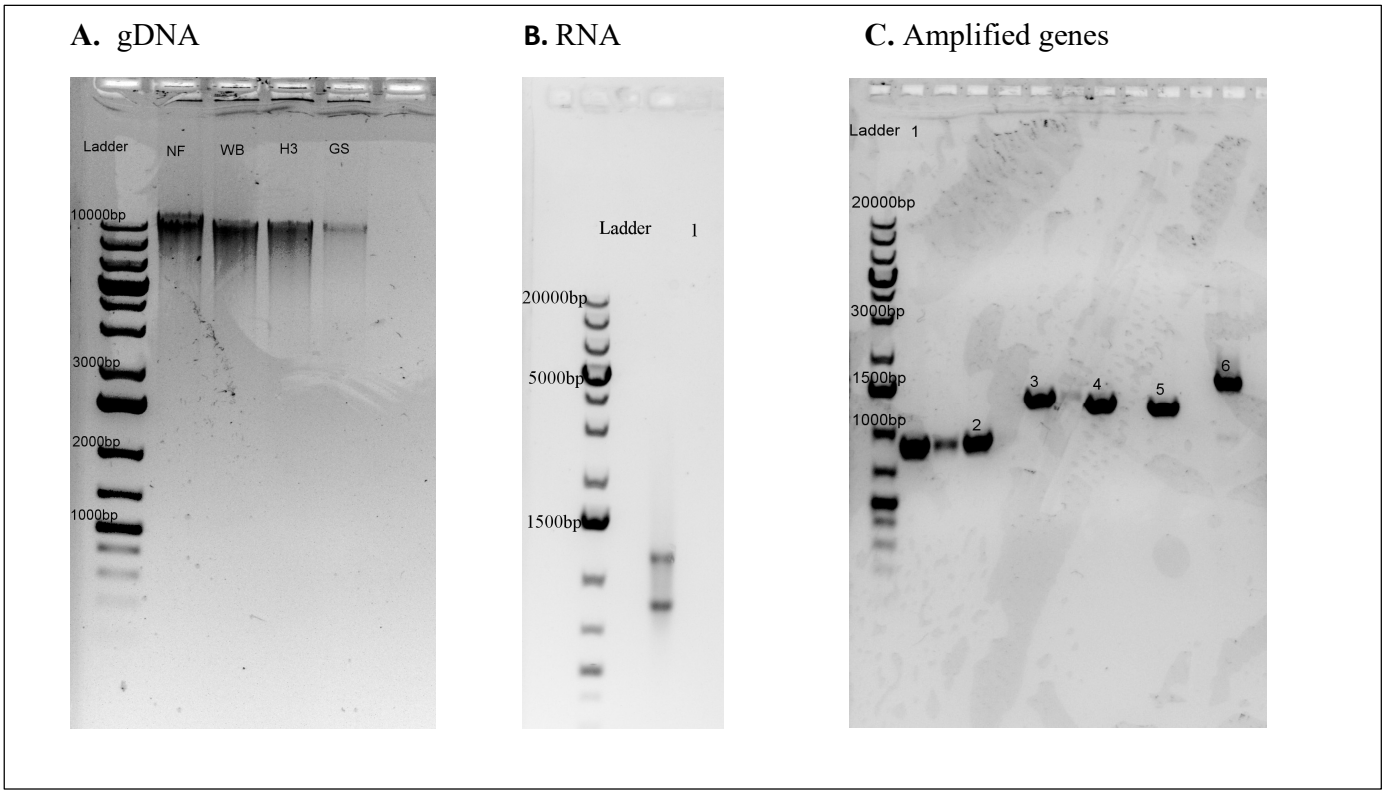
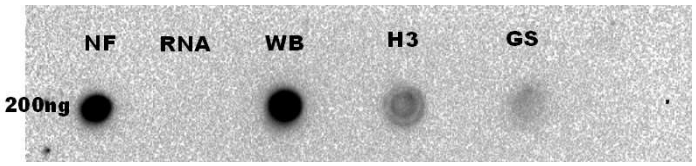
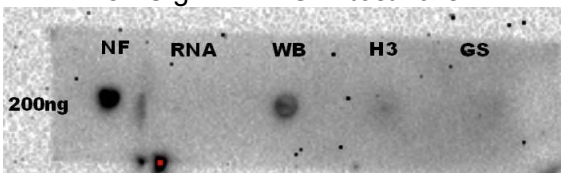


Figure 1. 1% TAE agarose showing A. 1kb plus ladder and RNA extracted from WB strain of Giardia. B. DNA (approx. 13mb) of different strains of Giardia (1kb ladder, 1-NF strain, 2-H3 starin, 3-WB strain, 4-GS strain) C. amplified genes lkb ladder, **1.**GL50803 1902 880bp, **2.**GL50803 1903 888bp,

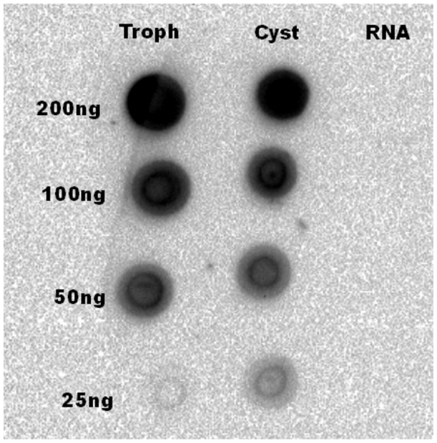
A. α6mA in gDNA of *G. intestinalis* strain



B. α5mC gDNA of *G. intestinalis* strain



C. α6mA methylation in gDNA of WB strain



D. α5mC methylation in gDNA of WB strain

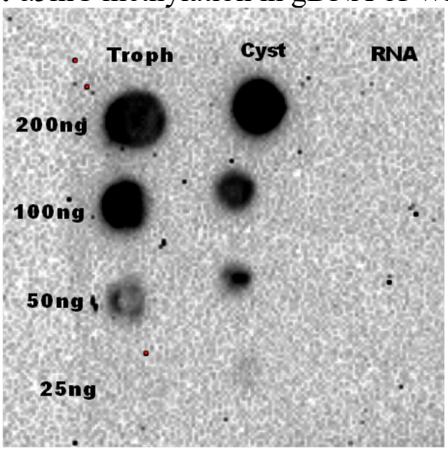


Figure 2. Dot blot showing methylations in different strains of G. intestinalis. Detection of methylation in both trophozoites and cyst of WB strain. (A) 6mA methylation of Assemblage A and B. (B) 5mC methylation in assemblage A and B. (C) 6mA methylation in WB trophozoites and cysts with dilution

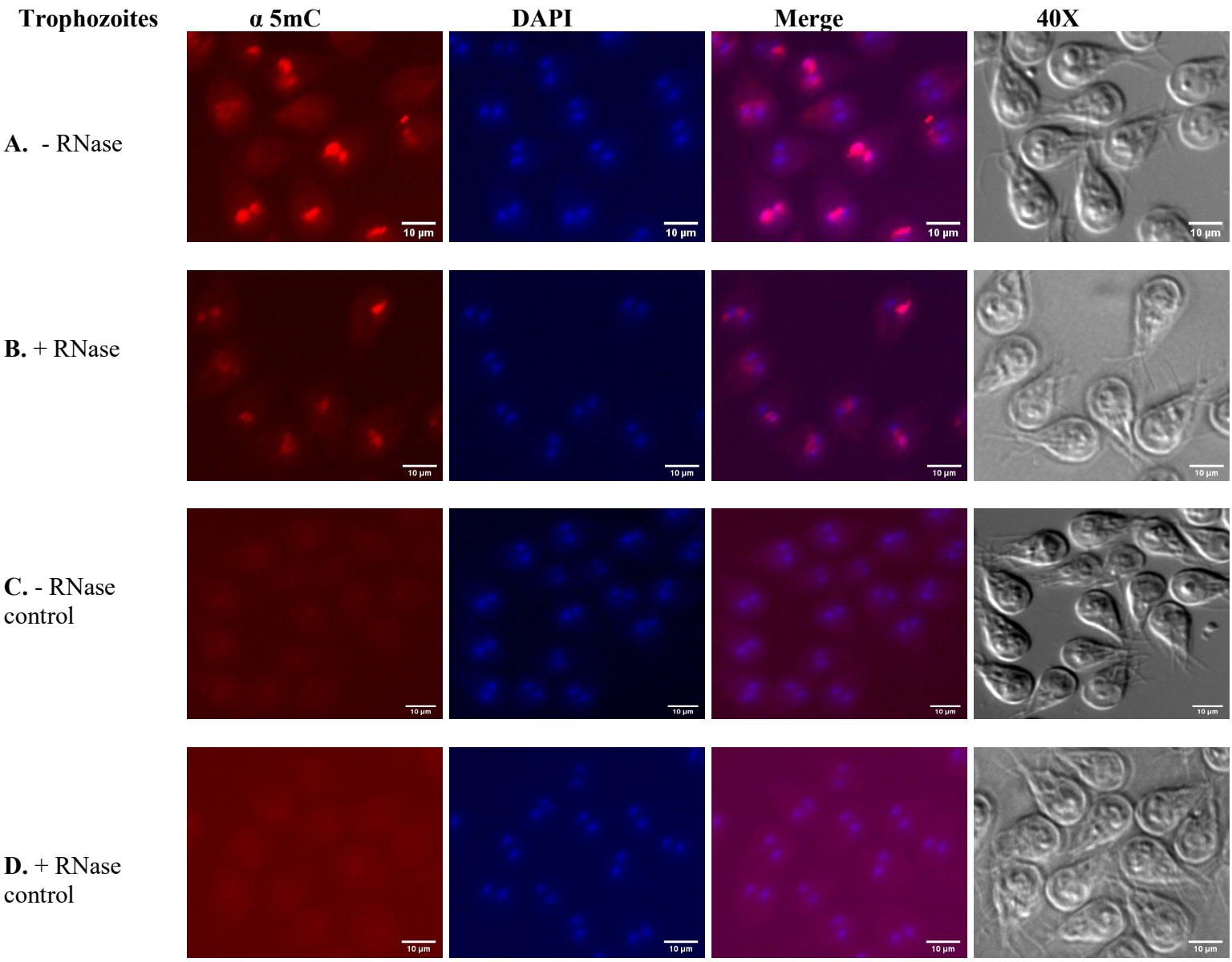


Figure 3. Immunofluorescence assay using α5mC antibody (red) detects 5mC methylation in the nucleus of *G. intestinalis* trophozoites, nuclei were stained with DAPI (blue). Images were taken by Nikon eclipse Ti imaging fluorescence microscope and analyzed by ImageJ 1.53e (Java 1.8.0_172. 64-bit). (A) trophozoites without RNase treatment. (B) trophozoites with RNase treatment. (C) control (fluorescently labelled secondary only) of trophozoites without RNase treatment. (D) control of trophozoites with RNase treatment.

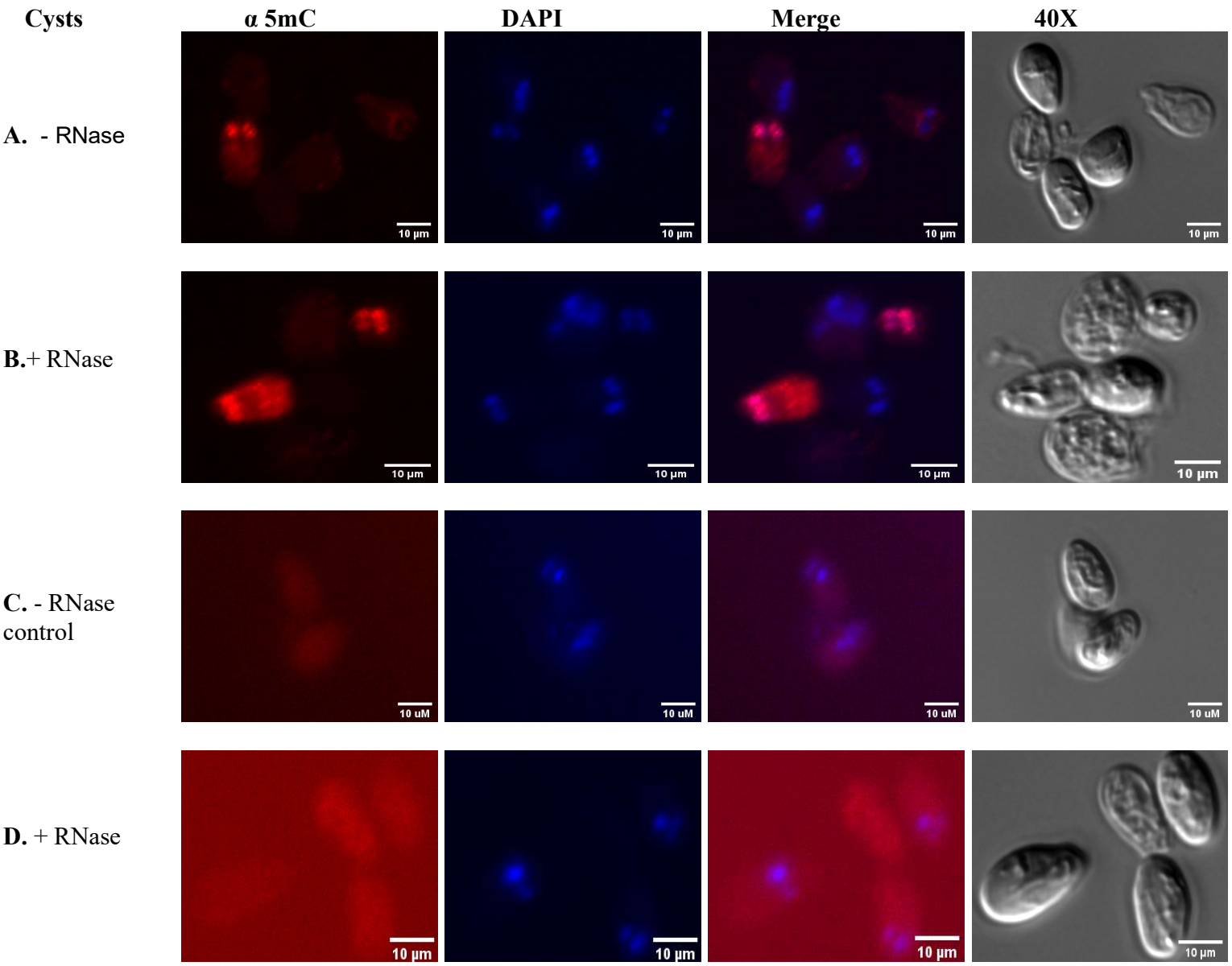


Figure 4. Immunofluorescence assay using α5mC antibody (red) detects 5mC methylation in the nucleus of *G. intestinalis* cyst, nuclei were stained with DAPI (blue). Images were taken by Nikon eclipse Ti imaging fluorescence microscope and analyzed by ImageJ 1.53e (Java 1.8.0_172. 64-bit). (A) Cysts with no RNase treatment. (B) cysts with RNase treatment. (C) control of cysts (fluorescently labelled secondary only) with no RNase treatment. (D) control of cyst with RNase treatment.

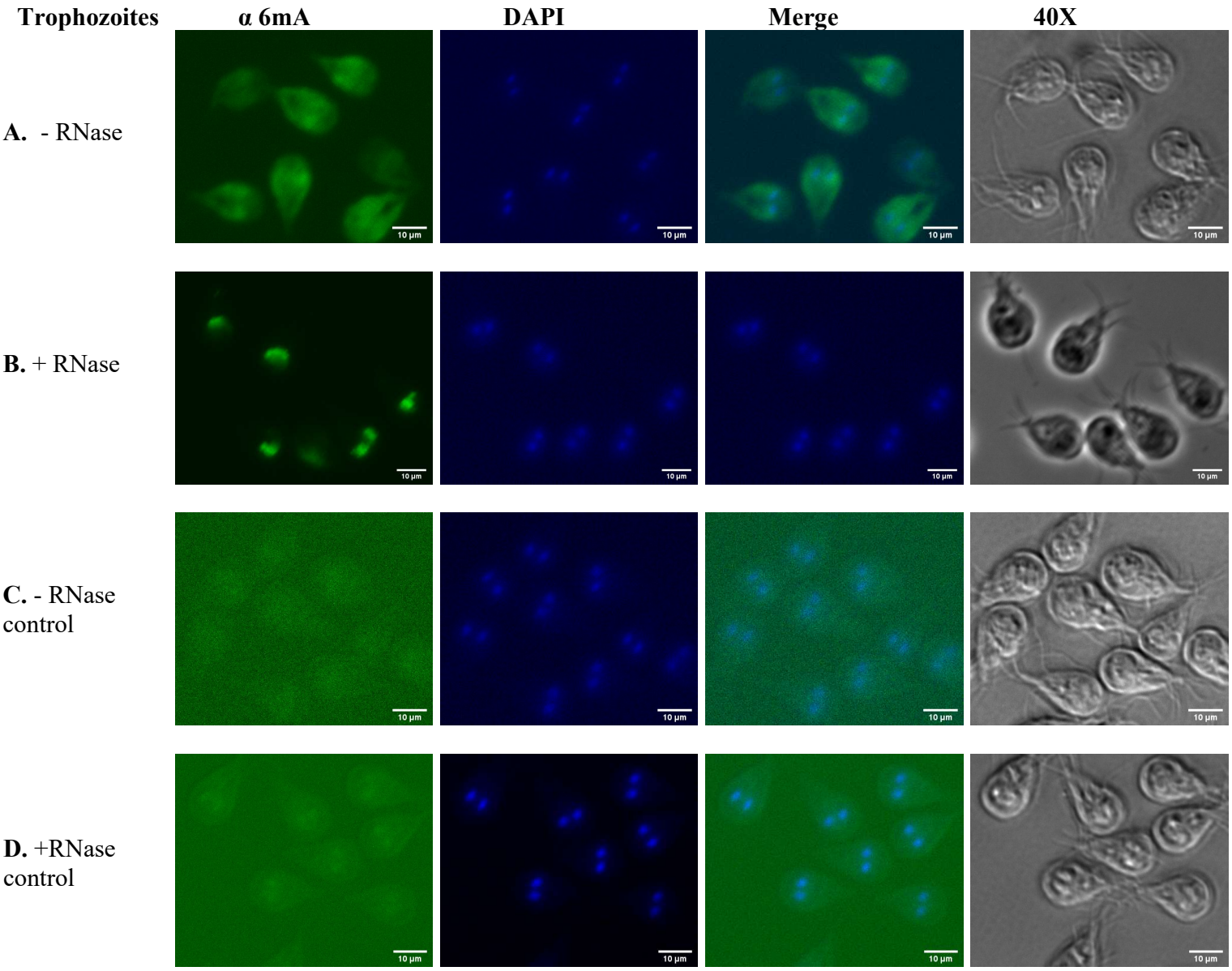


Figure 5. Immunofluorescence assay using α6mA (green) antibody detects 6mA methylation in the cytoplasm and nucleus of *G. intestinalis* trophozoites, nuclei were stained with DAPI (blue) Images were taken by Nikon eclipse Ti imaging fluorescence microscope and analyzed by ImageJ 1.53e (Java 1.8.0_172. 64-bit). (A) trophozoites without RNase treatment. (B) trophozoites with RNase treatment. (C) control (fluorescently labelled secondary only) of trophozoites without RNase treatment. (D) control of trophozoites with RNase treatment.

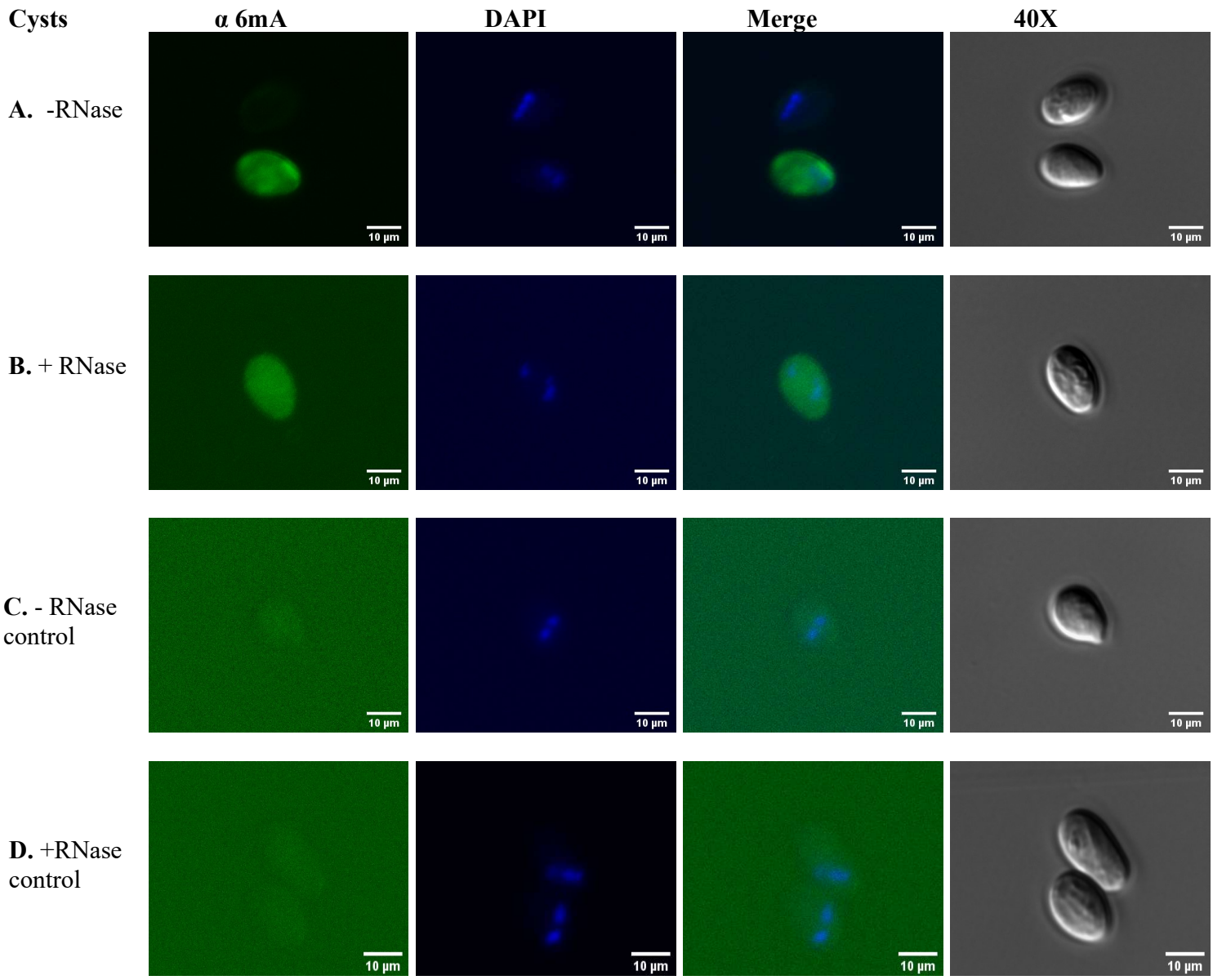


Figure 6. Immunofluorescence assay using α6mA antibody detects 6mA methylation in the nucleus of G. intestinalis cyst, nuclei were stained with DAPI (blue). Images were taken by Nikon eclipse Ti imaging fluorescence microscope and analyzed by ImageJ 1.53e (Java 1.8.0_172. 64-bit). (A) Cysts with no RNase treatment. (B) cysts with RNase treatment.

3.2 Identification and localization of putative methyltransferases in the WB strain of *G. intestinalis*

Our data suggest that there is DNA methylation in *Giardia*. DNA methylation has also been found in other pathogenic protozoa like *Plasmodium* and *Trichomonas*[27,39]. However, the enzymes introducting these modification have not been identified. In order to try to identify putative DNA methylases, we performed bioinformatic mining of the recently completed *Giardia* WB genome[40]. In total 59 genes in the WB genome encode proteins with domains that are similar to DNA or RNA methylases (Sup. File 2). The reason to look at both DNA and RNA methylases is that many RNA methylases also have some DNA methylase activity[41]. In the analysis we looked for structural similarities to known DNA methylases, expression levels during different conditions, conservation between assemblage A and B and also with another *Giardia* species, *G. muris*. We also modeled the structure of a selected number of putative DNA methylases to look for structural conservation. In the end, six putative methyltransferase genes in WB strain of *G. intestinalis* were chosen to be studied further by cloning the genes into the pPAC vector under the control of their promoters. The vector introduces a C terminal HA tag for localization studies by IFL(Table 3). The constructs were transfected into trophozoites, stained using anti-HA antibody, and localized by IFL (Figure 7).

GL50803_1902 is a small (219aa) hypothetical protein with an S-adenosyl-L-methionine (SAM) dependent methyltransferase domain (IPR029063) found on chromosome 5 down-stream of another putative DNA methyltransferase, GL50803_1903**,** in the WB isolate. It is syntenic with orthologs in the assemblage B isolate GS and assemblage E isolate P15. The gene is expressed at a constant level throughout encystation. Analysis of this protein using a profile Hidden Markov Model (HMMer) indicates that it has a methyltransferase domain weakly similar to release-factor glutamine methyltransferase in *Nitrareductuctor pacificus.* Structural modeling using iTASSER identified this protein as similar in structure to the 6hidA crystal structure of C21orf127, a human histone methylase in a complex with SAH, but again, the score is low. Thus, this is a small protein unique to *Giardia* with a methyltransferase-like domain. Localization of this protein indicated that the protein is mainly found in the nucleus but also in the cytoplasm of the parasite, as shown in Figure 7.

GL50803_1903 is another small protein (220aa) annotated as a DNA methyltransferase with a very low isoelectric point of 4.8. It is found on chromosome 5 together with GL50803_1902 in *Giardia*'s genome, and there are syntenic orthologues in assemblages B and E. Similar proteins can also be found in the other diplomonad parasites *G. muris* and *Spironucleus salmonicida*. The expression of this gene is relatively low, but it is expressed throughout the life cycle. Structural modeling using iTASSER indicates that the protein has structural similarity to 6hidA as above but also to the human m6A methylase N6 AMT1. We could not successfully localize this protein, even though the epitope-tagged protein was successfully transfected into the parasite (Figure 7). This can be due to over-expression problems since this is usually a protein with low expression in *Giardia*.

GL50803_8691 is a slightly larger, 39kDa protein annotated as a tRNA adenine methyltransferase subunit. There are syntenic orthologs in assemblage B and E isolates and similar proteins are found in *S. salmonicida* and *G. muris*. Transcriptome and proteome data show low expression throughout the life cycle. Structural modeling using ITASSER indicates that this protein has a structural similarity close to the 5CCB crystal structure of tRNA (adenine(58)-N(1))-methyltransferase catalytic subunit TRMT61A. Analysis using profile Hidden Markov Models (HMMer) also indicates that the protein has a SAM-dependent methylase domain and a GCD14 domain. The cloning of this gene failed.

GL50803_21512 is a 291aa basic protein annotated as a putative S-adenosylmethionine-dependent methyltransferase. This gene is also found on chromosome 5 in the *Giardia* genome. There are syntenic orthologs in assemblage B and E isolates, and similar proteins are present in *S. salmonicida* and *G. muris*. Transcriptome and proteome data show low expression throughout the life cycle. Structural modelling using iTASSER show structural similarity to human 18S rRNA (guanine-N(7))-methyltransferase and yeast putative methyltransferase BUD23. Biosequence analysis using profile Hidden Markov Model indicates that the protein has a SAM-dependent methyltransferase domain. Localization of these proteins in the WB strain of *G. intestinalis* using IFL showed that the protein is found in the nucleus as it completely overlaps with the DAPI, as shown in Figure **7**.

GL50803_100959 is a 323aa acidic, hypothetical protein. This gene is found on chromosome 4 in the WB assemblage A of *G. intestinalis*. There are syntenic orthologues in assemblages B and E. Similar proteins can also be found in the other diplomonad parasites *G. muris* and *Spironucleus*

salmonicida. The expression of this gene is relatively low but it is expressed throughout the life cycle. Biosequence analysis using profile hidden Markov model shows that the protein has a SAM dependent methyltransferase and lysin methyltransferase domains. iTTASSER modeling indicates that the protein has a structural similarity to the 4qpnA crystal structure of human protein-lysine methyltransferase METTL21D. We could not localize this protein in *G. intestinalis* since transfections failed.

GL50803_100887 is annotated as a 416 aa putative methyltransferase in the WB genome. It has not been localized to a specific chromosome, but it is very similar to the putative methyltransferase GL50803_103058 on chromosome 2 (2 internal amino acid changes). In another assemblage A, B and E *Giardia* isolates there are also two very similar or identical copies of this putative methylase. Even if it a multi-copy gene the expression levels are very low. Biosequence analysis using profile hidden Markov model shows that its protein has FtsJ domain. Structural modelling using iTASSER suggests that it is similar to the structure 4N48, human Cap-specific mRNA (nucleoside-2-O-)-methyltransferase 1. This protein localizes in the nucleus as shown in Figure 7.

Table 3 Selected genes, their annotation and structural domain generated from Giardia DB (www.giardiadb.org)

ORF	Annotation	Chromosome	Structural domain	Expression in vivo	Structure	organism	P value
GL50803_1902	Hypothetical protein	5	S-adenosyl-L-methionine-dependent methyltransferases	Lower	-	-	-
GL50803_1903	DNA methyltransferase	5	S-adenosyl-L-methionine-dependent methyltransferases hemK_rel_arch: putative methylase	Lower	-	-	-
GL50803_8691	tRNA methyltransferase subunit, putative	4	tRNA methyltransferase, GCD14 type	High	tRNA (adenine(58)-N(1))-methyltransferase catalytic subunit TRMT61A	Homo sapiens	1.6×10^{-36}
GL50803_21512	S-adenosylmethionine-dependent methyltransferase, putative	5	S-adenosyl-L-methionine-dependent methyltransferases	lower	Putative methyltransferase BUD23	Saccharomyces cerevisiae S288c	2.2×10^{-46}
GL50803_100887	Methyltransferase, putative	-	-	higher	Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	Homo sapiens	8.8×10^{-21}
GL50803_100959	S-adenosyl-L-methionine-dependent methyltransferases	4	S-adenosyl-L-methionine-dependent methyltransferases	Lower	Protein-lysine methyltransferase METTL21D	Homo sapiens	5.6×10^{-8}

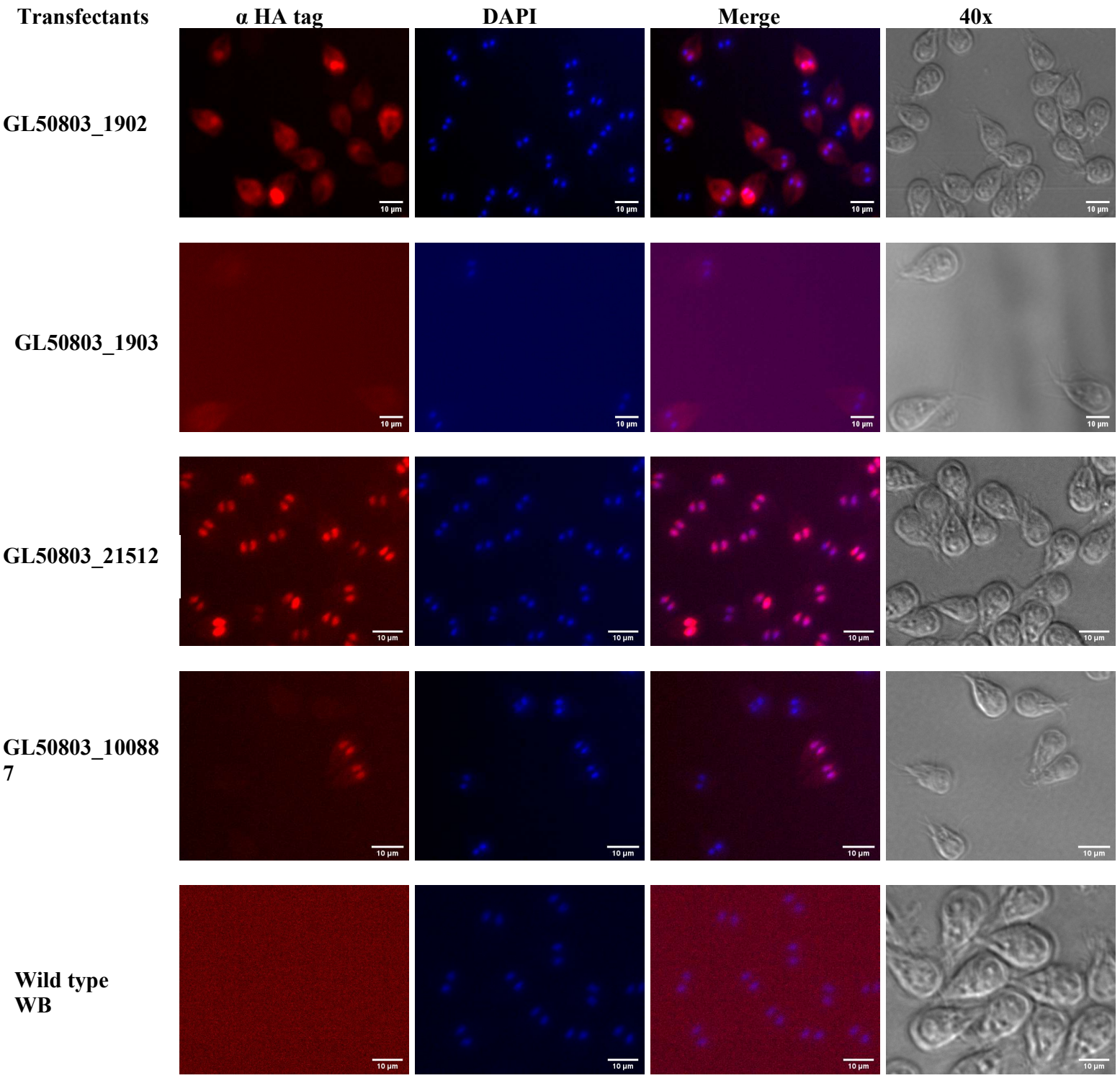


Figure 7. The localization of *G. intestinalis* methyltransferases. The localization of 3HA epitope-tagged methyltransferases by immunofluorescence microscopy. Transfectants were detected with a mouse anti Hemagglutinin (HA) antibody conjugated to Alexa Fluor 594 (red), nuclei were stained with DAPI (blue). Images were taken by Nikon eclipse Ti imaging fluorescence microscope and analyzed by ImageJ 1.53e (Java 1.8.0_172. 64-bit).

3.3 Screening of methylation targeting drugs active against *Giardia*

One major quest of this project was to detect methylation in *Giardia*. The other primary goal was to screen if methylation targeting drugs can be used as an alternative treatment for giardiasis. New drugs are needed due to the problem of slowly increasing resistance of the current treatment options[9]. To test these drugs, we challenged *G. intestinalis* trophozoites from the WB strain with two different methylation targeting drugs: 5-aza-2'deoxycytidine and 5-azacytidine. We established the drug concentrations needed to inhibit the cells by 50% (IC_{50}). 5-aza-2' deoxycytidine has a significant effect on the parasites (Table 4), generating an IC_{50} of 1.46±0.14 µM. Thus, the IC50 is lower than the currently used drug metronidazole that has an IC50 of 5 µM[42]. On the contrary, 5-azacytidine is less effective (Table 4), with an IC50 value of 111±24 µM.

Table 4. Percentage survival of Giardia intestinalis after treatment with different concentrations DNA inhibitors

DNA inhibitor	1 µM	2 µM	5 µM	10 µM	20 µM	50 µM	10 0µM	IC_{50}	STDV
% 5-aza-2'deoxy cytidine	81.32507	57.29268	7.842925	5.24623	2.770809	1.38013	1.058806	2.13	± 0.03
% 5-azacytidine	100.7567	92.24101	103.7833	92.87488	90.07389	76.54504	50.1629	104	± 2.89

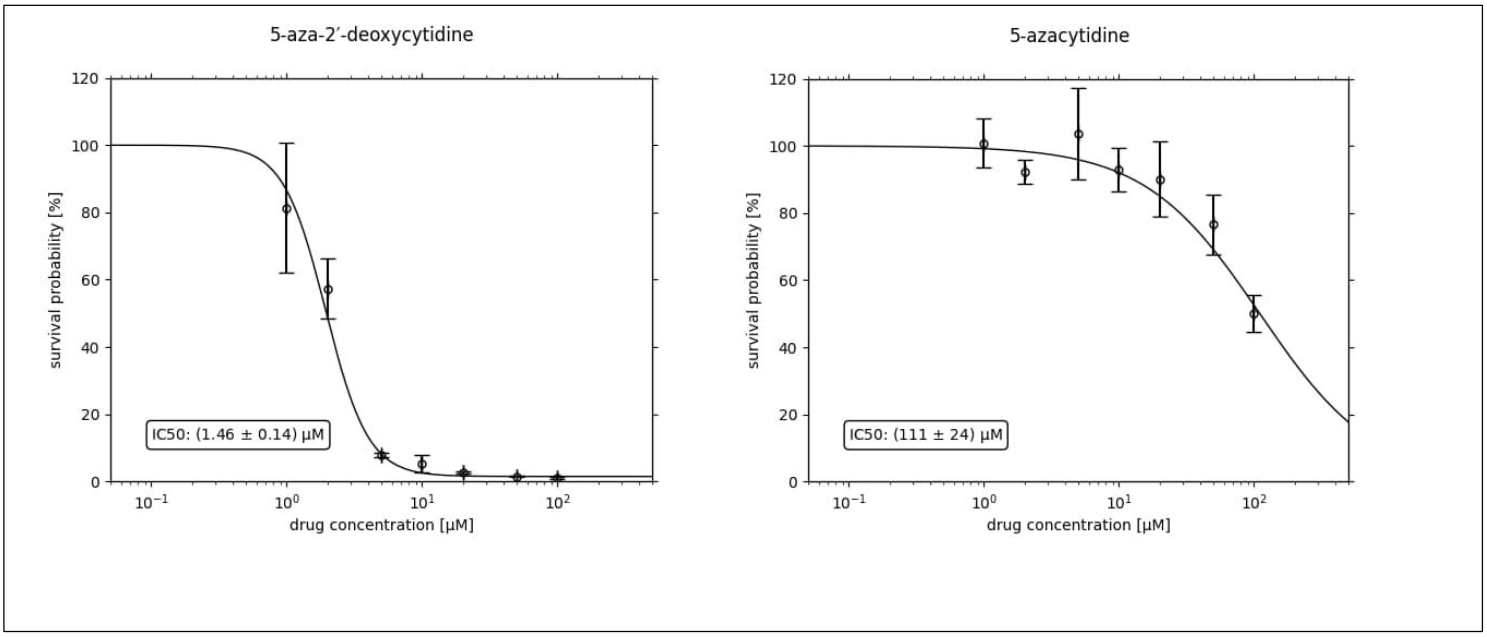


Figure 8. IC50 of 5-aza-2 deoxy cytidine and 5-azacytidine on G. intestinalis WB strain

Chapter 4. Discussion

Epigenetic regulation is essential in most organisms, but it differs in what molecules are affected, the responsible mechanism, and its downstream effects. One example is methylation of RNA and DNA. Modifications on DNA and RNA includes 5mC and 6mA but are not limited to these. 5mC methylation on DNA in prokaryotes mainly function to protect their genetic material from external DNA[15].5mC modifications on DNA is known to be important for imprinting and silencing of X chromosomes[16]. It has been reported by Li *et al*., 2019 that a large number of eukaryotic cells possess 6mA modifications on DNA and several of this modification on different types of RNA, but their functions are not yet explored in many of these organisms[43]. 6mA modifications on both DNA and RNA requires the deposition of a methyl group on the nitrogen on the 6th position of adenine ring but are catalyzed by different methyltansferases[43]. 6mA modification on DNA has been reported to regulate mitochondrial stress adaptation in *C. elegans*[44]. 6mA modifications on RNA is said to determine the fate of a transcript by regulating rapid transition in gene expression programs in embryonic stem cells[45]. It has also been reported that *Plasmodium falciparum* uses 6mA modification of RNA to regulate its virulence[25]. Epigenetic regulation has been shown to be important in *Giardia*. Histone methyltransferase I catalyzes the addition of methyl groups to lysine and arginine residue and has been identified to regulate encystation in *Giardia*[46]. Acetylation of H4K8 and increase in H4K16 are critical to encystation whiles a decrease in acetylation of H4K8 and methylation of H3K9 is important for antigenic variation in *Giardia*[47]. But methylation on the genomic DNA of *Giardia* has not yet been reported. This work, as well as previous unreported data generated by the group using PacBio sequencing revealed that there are 6mA, 5mC, and most likely 4mC in the genomic DNA, and that there exist many other modifications on RNA[48]. We detected 6mA and 5mC DNA methylation in trophozoites and cyst of *G. intestinalis* using an immunofluorescence assay complemented with the dot blot technique. Figure 5 shows both cytoplasmic and nuclear localization of 6mA modifications, suggesting that there is 6mA modification on both RNA (Figure 5A) and DNA (Figure 5B) in *G. intestinalis.* In contrast, we showed by dot blotting that the antibody did not react with purified total RNA, (Figure 2). However, the same antibody seems to bind RNA in the cytoplasm as shown in Figure 5. The reason for this contradiction might be that the extracted RNA contains a high amount of ribosomal RNA, and only about 1-3% mRNA. Thus, since we expect that most of the 6mA modifications in eukaryotic RNA exist in mRNA, the amount of mRNA in the extracted total RNA might be too little to be detected. This also aligns with the fact that the 6mA signals from the cytoplasm are strong and ribosomal RNA is mainly localized on the ER and in the nuclei. It could also be that the IFL is more sensitive than dot blotting. The 6mA modification might also localize at a region of the DNA that is concealed or surrounded by RNA or other proteins as evaluated by Zhong *et al.*,[49]. Treatment with RNase degrades RNA molecules that are concealing the sites resulting in a stronger signal in Figure 5. We were not able to come to a conclusion about 6mA methylation in the cyst, because it has a hard cyst wall protein[6], which makes it difficult to permeate. Therefore, the polyclonal 6mA antibody could not penetrate it and remain stuck on the cell wall, resulting in the signal generated in Figure 6. 5mC modification is detected in the nucleus of trophozoites and there is no true cytoplasmic signal but when the trophozoites were treated with RNase, the signal tends to diminish or be present in only one of the nuclei of the parasite (Figure 3). We do not know why, but this could arise based on a number of reasons; first of all we are not dealing with a synchronized population of cells, secondly, the RNase may have some partial effect on the DNA and thus degrade the DNA, making the signal to weaker, thirdly, the antibody based method produces some additional false signal as evaluated by Singh *et al.,* [50], fourth, it could be that 5mC

is methylated on RNA in the nucleus and treatment with RNase degrades the methylated RNA and leaves only DNA methylated which makes the signal diminish. The latter is less likely in the case of *Giardia* because treatment with nucleoside analogue 5-azacytidine that incorporates into RNA[34] has a high IC50 value(Figure 8**)**. On the other hand, there are 5mC modifications on DNA in both RNase treated and untreated cysts since the signal is completely colocalizing with the nucleus in both cases Figure 4. This revealed that 5mC methylations might be necessary for the suppression of several genes since the cyst is the metabolically dormant form of the parasite[6]. Unreported previous data showed that 5mC is not abundant in the gDNA of *G. intestinalis*.

On the path to detecting methylations in *G. intestinalis*, we used a set of bioinformatic analyses to classify putative DNA methyltransferases in sequenced *Giardia* genomes to look for enzymes that might be responsible for adding a methyl group to these genetic molecules. It should be noted that there are very few characterized DNA methyltransferases in eukaryotic cells and when they were queried against the *Giardia* genomes only weak hits were obtained. Thus, alternative approaches are needed. In this work, six putative DNA methyltransferases were identified and cloned into our *Giardia* expression vector pPAC. Three of the putative DNA methylases (GL50803_1902, GL50803_21512 and GL50803_100887)Figure 7 colocalize with the nuclei, as would be predicted of DNA methyltransferases. The protein encoded by ORF GL50803_1902 is a small protein unique to *Giardia* with a SAM-dependent methyltransferase-like domain. It localizes to the nucleus with stronger signals compared to signals in the cytoplasm (Figure 7), but the activity cannot be predicted from sequence analyses alone. A search in a protein structure data base (PDB) for the structural similarity of GL50803_21512 protein sequence had a hit on 6G4W, a cryo-EM structure of a late maturation state of the human pre-40S ribosomal subunit[51]. The location of this protein to the ribosome aligns with the prediction by ITASSER that shows homologies to Bud23-TRM112, which is associated with m(7)G methylation during ribosome small sub unit biogenesis[52]. As described by Létoquart *et al* the role m(7)G methylation of premature ribosomal subunits is not completely understood, but it is known not to have an effect on cell growth, whereas depletion of the BUD23 protein has an effect on growth[52]. BUD23 has recently been shown to promote the release of factors surrounding its binding site to induce structural rearrangements during the progression of the small subunit (SSU) Processome[53]. The function of GL50803_21512 in *Giardia* could be related to ribosome biogenesis and RNA modifications but it remains to be shown. Profile HMMer searches revealed that the GL50803_100887 protein contains a ftsJ domain, this domain is reported to be part of proteins methylating 23S rRNA and vital for cell growth[54]. The gene GL50803_1903, encoding a small protein with a SAM-dependent methyltransferase domain, was cloned, and transfected into *Giardia,* but we were unable to detect any signal from the fusion protein. It could be due to that the protein is expressed in very low amounts and thus not detectable. The transfections with GL50803_100959 and GL50803_8691 failed, which might be because overexpression of these proteins is toxic to the cells.

In this study, we tested the activity of two different drugs affecting DNA methylation on *Giardia* trophozoites. The two drugs are nucleoside analogs; 5-aza 2'-deoxycytidine incorporates into newly synthesized DNA in the S-phase of the cell cycle and replaces cytosine forming aza-cytosine-guanine dinucleotides[34]. These are recognized by DNA methyltransferase as natural substrates, resulting in the inhibition of the DNA methyltransferases, hypomethylation and growth arrest[34]. The drug is effective against the *Giardia* parasite with an IC50 value of 2 μM.

This strengthens our conclusion that the DNA is methylated in *Giardia* and that interference with this is lethal to the parasite. Unpublished data in the Svärd group have shown that if the drug is added to a population of encysting cells, encystation efficiency is reduced, and very few mature cysts are formed. This shows that 5-aza 2'-deoxycytidine or related derivatives with less toxicity could be alternatives to *Giardia* treatment.

5-azacytidine, which instead incorporates to 80-90% into RNA[34] has little effect on the *Giardia* parasite Figure 8 . In human cells, 10-20% of 5-azacytidine is converted to 5-aza-dCDP after multiple steps by ribonucleotide reductase and the 5-aza-dCDP is then incorporated into DNA, blocking cell growth. *Giardia* has no ribonucleotide reductase enzyme, which probably reduces the efficacy of the drug in *Giardia*. 5-azacytidine treatment results in very high IC_{50} value (>100 μM) for *Giardia*, and since the pathway of 5-azacytidine is mainly via incorporation into RNA[37], in the end affecting translation, it suggests that this type of modification does not really have a significant effect on the survival of the parasite. With this analysis we can indicate that methylation on DNA is important for the survival of the parasite and that 5mC methylation on the RNA may be less important for the survival of the parasite. It could also be that there is no or little 5mC methylations on RNA, which corresponds to our experimental results from dot blots and immunofluorescence. 5mC modification of DNA is known to play an important role in the proliferation and differentiation of embryonic stem cells[55], and that 5-aza2'-deoxycytidine treatment results in inhibition of cell proliferation[34]. We can therefore say based on the reaction of *Giardia* to 5-aza-2'deoxycytidine that 5mC methylation on gDNA of *Giardia* plays a crucial role in proliferation, differentiation and survival in a stressful environment.

In conclusion, we have demonstrated 5mC and 6mA methylation on genomic DNA and 6mA on RNA in the WB strain of *Giardia intestinalis*. The 5mC modifications plays an essential role in the proliferation of the cells and differentiation from trophozoites to cyst. We still do not know what enzymes introduce the modified nucleotides in *Giardia* but we have a few candidates that will be further characterized.

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Ọpẹ ni fun Ọlọrun Elédùmarè.

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