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Specific histone modifications play critical roles in the control of encystation and antigenic variation in the early-branching eukaryote *Giardia lamblia*

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Highlights

- The early-branching eukaryote *Giardia lamblia* shows the most common histone marks.
- Histone deacetylation is particularly relevant for *Giardia* differentiation and adaptation.
- NAD^+-dependent and NAD^+-independent deacetylases regulate parasite differentiation.
- Epigenetic marks control *Giardia* gene expression for survival inside and outside its hosts.

Abstract

During evolution, parasitic microorganisms have faced the challenges of adapting to different environments to colonize a variety of hosts. *Giardia lamblia*, a common cause of intestinal disease, has developed fascinating strategies to adapt both outside and inside its host´s intestine, such as trophozoite differentiation into cyst and the switching of its major surface antigens. How gene expression is regulated during these adaptive processes remains undefined. *Giardia* lacks some typical eukaryotic features, like canonical transcription factors, linker histone H1, and complex promoter regions; suggesting that post-transcriptional and translational control of gene expression is essential for parasite survival. However, epigenetic factors may also play critical roles at the transcriptional level. Here, we describe the most common post-translational histone modifications; characterize enzymes involved in these reactions, and analyze their association with the *Giardia*'s differentiation processes. We present evidence that NAD^+-dependent and NAD^+-independent histone deacetylases regulate encystation; however, a unique NAD^+-independent histone deacetylase modulate antigenic switching. The rates of acetylation of H4K8 and H4K16 are critical for encystation, whereas a decrease in acetylation of H4K8 and methylation of H3K9 occur preferentially during antigenic variation. These results show the

complexity of the mechanisms regulating gene expression in this minimalistic protozoan parasite.

Abbreviations: ORF: Open Reading Frame; CWPs: Cyst Wall Proteins; HDAC: Histone Deacetylase; HAT: Histone Acetyl Transferase; IFA: Immunofluorescence Assay; VSP: Variant-specific Surface Protein; mAb: monoclonal Antibody; qPCR: Quantitative Polymerase Chain Reaction; ChIP: Chromatin Immunoprecipitation; VSG: Variable Surface Glycoprotein.

Keywords

Histone marks; cellular differentiation; gene regulation, histone methylases; histone deacetylases; HDAC; Sirtuin.

1. Introduction

Giardia lamblia is a non-invasive parasite that inhabits the upper small intestine of humans (Adam, 2001). This protozoan belongs to the earliest diverging branch of the eukaryotic line of descent and is considered an excellent model to study evolutionary aspects of basic biochemical and cellular processes (Lujan and Svärd, 2011). *Giardia* possesses a small and compact genome of ~12 Mb containing nearly 9,000 ORFs (Morrison et al., 2007). As other protozoa, *Giardia* lacks canonical transcription factors and other gene regulatory elements (Carranza and Lujan, 2010). It is possible that some of these molecules escape bioinformatic identification due to their primitive condition or, more likely, epigenetic factors may be playing essential roles in the regulation of gene expression by altering chromatin structure (Croken et al., 2012).

Giardia presents a simple life cycle: the environment-resistant cysts and the disease-causing vegetative trophozoites (Adam, 2001). Between these developmental stages *Giardia* needs to rapidly adapt to establish an infection in a new host. *Giardia* differentiation into cysts (encystation) consists of the developmentally-regulated expression, transport and assembly of cyst wall proteins (CWPs) and a particular carbohydrate polymer (Lujan et al., 1997). The *Giardia* cyst wall protects the parasite from the harsh environmental conditions it has to endure outside the host's intestine (Adam, 2001; Lujan et al., 1997). When a putative host ingests water or food contaminated with *Giardia* cysts, a signal transduction cascade triggered by the low pH of the stomach allows the release of trophozoites in the upper small intestine (Ellis et al., 2003). Once in the small intestine, trophozoites produce a symptomatic infection or remain unnoticed (Adam, 2001).These events require a high degree of coordination to avoid the release of the trophozoites inside the stomach (which could be detrimental) or in the large intestine, where the nutrient levels turn the infection unlikely (Lujan et al., 1997).

Antigenic variation is a mechanism that allows parasite evasion from the host's immune response, enabling the establishment and maintenance of chronic and/or recurrent infections (Deitsch et al., 2009). Antigenic variation in *Giardia* consists of a clonal phenotype variation involving surface antigens called Variant-specific Surface Proteins or VSPs (Nash, 2002; Prucca et al., 2011). *Giardia* possesses approximately 200 different VSP genes (Adam et al., 2010; Morrison et al., 2007), but only one VSP is expressed at any point in time on the surface of individual trophozoites (Nash, 2002). Changes in the VSP expression also occur during the encystation process (Svärd et al., 1998; Carranza et al., 2002; Einarsson et al., 2016). We have previously shown that the regulation of VSP expression is controlled post-transcriptionally by an RNAi-like mechanism (Prucca et al., 2008). Additionally, Wang's group showed that miRNAs could be implicated in the control of VSP expression at the translational level (Saraiya et al., 2011).

Why just one VSP mRNA bypasses this silencing mechanism, accumulates in the cytoplasm, and is finally translated into the protein that will be expressed on the trophozoite surface remains an open question (Prucca et al., 2011). How *Giardia* switches the expressed antigen to another from its genomic repertoire is also unknown (Prucca et al., 2008). Nevertheless, early nuclear run-on experiments, which demonstrated that many VSP are simultaneously transcribed, also showed variations of the transcription level of individual VSP genes (Prucca et al., 2008), suggesting that the concentration of each VSP transcript may influence the selection of the one that avoids RNAi degradation (Prucca et al., 2008). Accordingly, it can be assumed that epigenetic factors may be involved in such regulation. However, how changes in chromatin influence gene expression during encystation and antigenic variation has not been studied in detail (Kulakova et al., 2006; Sonda et al., 2010).

Epigenetic factors influence the local chromatin architecture allowing or preventing the

accessibility of gene expression factors. Some of the best known epigenetic mechanisms are methylation of DNA and post-translational modification of histones (Kouzarides, 2007). *Giardia* has two copies of the histones H2A, H2B, H3 and three of H4, but no putative homologue of the linker histone H1 has ever been found (Wu et al., 2000; Yee et al., 2007). Just a few histone modifications have been mentioned in earlier reports (Dawson et al., 2007), but no broad studies have been performed regarding these aspects of the *Giardia* biology.

Among post-translational modifications of histones, acetylation and methylation of lysine residues in histone H3 and H4 are critical to the regulation of chromatin structure and gene expression (Vaquero, 2009). The steady-state levels of acetylation depend on the activity of histone acetyltransferases (HAT) and histone deacetylates (HDAC). According to the requirement of the cofactor nicotinamide adenine dinucleotide (NAD^+), histone deacetylases can be classified into NAD^+-independent deacetylases (or simply HDAC) and NAD^+-dependent deacetylases (Sirtuins or Sir2-like, for *Silent* Information Regulator 2) (de Ruijter et al., 2003; Frye, 2000; Yang and Seto, 2008). The HDACs have important roles in DNA repair and replication, cell cycle control, apoptosis and other functions (de Ruijter et al., 2003; Yang and Seto, 2008). Sirtuins have different functions, substrates, and subcellular localizations (Frye, 2000; Vaquero, 2009). Sirtuins modify chromatin structure through deacetylation of histones, particularly H4K16ac (Vaquero et al., 2007). Recently, histone deacetylases have gained considerable attention because of their function as metabolic sensors, mediating environmental stress responses (Fulco et al., 2003; Vaquero and Reinberg, 2009). This link between the environment and chromatin modifications allows detection of rapid environmental changes and effective adaptive processes, such as those that *Giardia* confronts during its life cycle.

In this work, we characterized the presence of the most common eukaryotic histone modifications associated with euchromatin, heterochromatin and intermediate stages during

Giardia proliferation and differentiation. We also characterized, localized, and inhibited two protein families with deacetylase activity: the NAD^+-independent HDAC and the NAD^+-dependent Sirtuins, reproducing and complementing earlier reports (Sonda et al., 2010). Additionally, we determined that specific histone deacetylation/methylation events play critical roles during encystation and antigenic variation of this important human parasite.

2. Materials and methods

2.1 Bioinformatics Analysis.

Known histone deacetylase domains were used to search homologous sequences using GenBank™ and the GiardiaDB (www.giardiadb.org/giardiadb). The identification of nuclear localization sequences was made using NLS Mapper (http://nls-mapper.iab.keio.ac.jp/), EPipe server (http://www.cbs.dtu.dk/services/EPipe/), and PSORT II (http://psort.hgc.jp/). For phylogenetic analyses, multiple alignments (MUSCLE) were used into the SeaView. Phylogenetic trees were computed using PhyML.

2.2 G. lamblia cultures and treatment with deacetylase inhibitors.

Trophozoites of isolate WB, clone 1267, clone 9B10 and clone A6 were cultured in TYI-S-33 medium (Keister, 1983). Encystation of trophozoites was induced as described (Lujan et al., 1996). For antigenic variation experiments, a *Giardia* clone expressing a unique VSP was obtained by serial dilution and selection by immunofluorescence assays (IFAs) using mAb that recognizes specific VSPs. Antigenic variation was induced according to Torri *et al*. (unpublished data). Monolayers of trophozoites were induced either to encyst or to switch in the presence of different concentrations of Trichostatin A, Sodium Butyrate or Nicotinamide. Harvested cells were used for IFA or lysed in RIPA buffer for 30 min and quantified for subsequent immunoblotting.

2.3 Histone immunoblotting and peptide competition assays.

Histones were isolated using the classical acid extraction method (Shechter et al., 2007). Many anti-histone modification antibodies from different sources were tested. Only those obtained from Abcam (anti-H3K4m1, anti-H3K4m2, anti-H3K4m3, anti-H3K9m3, and anti-H3K9ac) and from Millipore (anti-H4K8ac, anti-H4K16ac) showed a good level of detection and clear specificity. In all cases, each antibody was pre-incubated with a peptide that has the corresponding amino acid modification prior to the immunoblotting assays. The immunoblotting experiments were run in duplicate: one with the antibody pre-incubated with the corresponding peptide and another with a control antibody not pre-incubated with the peptide. The presence of negative bands in the pre-incubation condition and of reactive bands in the non pre-incubation conditions confirmed the specificity of each antibody. Peptides used were H3 peptide - mono methyl K4, H3 peptide - dimethyl K4, H3 peptide - trimethyl K4, H3 peptide - trimethyl K9, H3 peptide - acetyl K9 from Abcam, and Acetyl-Histone H4 (Lys8) and Acetyl-Histone H4 (Lys16) peptides from Millipore.

2.4 Transfection of Giardia HDAC and Sirtuins.

The plasmid pTubHApac that fused three Influenza Hemagglutinin (HA) epitopes to the C terminus of the expressed protein was used (Prucca et al., 2008). Trophozoites were transfected with the corresponding construct by electroporation and selected entire coding region of the ORF3281 (Gene ID: GL50803_3281); ORF10707 (Gene ID: GL50803_10707); ORF10708 (Gene ID: GL50803_10708); ORF11676 (Gene ID: GL50803_11676); ORF16569 (Gene ID: GL50803_16569); ORF6942 (GL50803_6942) were amplified from *Giardia* genomic DNA (isolate WB/clone 1267) by PCR using the following forward (FO) and reverse (RO) oligonucleotides containing different restriction sites: ORF3281, restriction site for NcoI/EcoRV, FO: 5′-atgCCATGGCGCCTTCAAAACCCTC-3′; RO: 5′-

gcgGATATCGTTCTCATCTAACCCCGCTTCACT-3´; ORF10707, restriction site for ApaI/SmaI, FO: 5′-cattGGGCCCAAATCGGCTCTCGAAGCCAT -3′; RO: 5′-tccCCCGGGCTGGCGCGATCGGGG-3′; ORF10708, restriction site for ApaI/SmaI, FO: 5′-attGGGCCCCCTGCTAATTATGGGCAGACTC-3′; RO: 5′-tccCCCGGGTTTACCTGAAGGCTCATCAGTAAG-3′; ORF11676, restriction site for ApaI/EcoRV, FO: 5′-cattGGGCCCCAGATAGAAGAGGACAGACGG-3′; RO: 5′-gcgGATATCGGTAACGCGCTGATG -3′; ORF16569, restriction site for NcoI/EcoRV, FO: 5′-atgCCATGGTACCCGATGTAGATGTCAT-3′, RO: 5′-gcgGATATCATCTATATTGAGAATAGACGATCCAAT-3′; ORF6942, restriction site for ApaI/EcoRV, FO: 5′-cattGGGCCCAATGTGTATGAGCGCTTAGCT-3′, RO: 5′-gcgGATATC ATATGGGATATTTAGAATGTTTGC-3′.

2.5 Immunofluorescence Assays.

Trophozoites cultured in either growth or encystation media were subjected to IFAs as reported (Prucca et al., 2008). Primary anti-HA, anti-histones, anti-CWPs or anti-VSPs antibodies were used. The mAb 7D6 recognizes CWP2. The mAbs 5C1, 6E7 and 9B10 recognize the VSPs1267, VSPA6 and VSP9B10, respectively. Specimens were viewed on a Leica IRBE fluorescence microscope using a PL APO 100x/1.40-0.70 Oil 0.17/D objective and images taken with a Hamamatsu ORCA ER-II camera. Controls included omission of primary antibody and staining of untransfected cells. Images were processed with the Image J software. Regarding histone modifications, variation in localization and intensity of the marks were evaluated microscopically and quantified using the Image J software. Digital quantification always correlated with the visual observations.

2.6 Chromatin immunoprecipitation (ChIP).

ChIP assays were performed by using a commercial kit (ab500, Abcam). Briefly, trophozoites cultured either in growth or encystation medium, in the presence or not of the different inhibitors, were used. The cells were fixed in 1% formaldehyde before lysis. Chromosomal DNA was shared using a sonicator to an optimal DNA fragment size of 200–1000 bp. Chromatin was precipitated using 2 µg rabbit pAb against different histone modifications and a rabbit anti–mouse IgG was used as isotype control.

2.7 Real Time PCR.

Quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR kit (Qiagen). We used 1 µl of the ChIP product (or 1µl of cDNA in the corresponding case) in a final volume of 25 µl; a triplicate for each gene was performed. RNA was standardized by quantification of glutamate dehydrogenase (GDH) gene as reference. Experiments were performed in triplicate. We studied the 5´ UTR regions of the CWP2, VSP1267 and VSP9B10 and coding regions of the same genes using the following forward (FO) and reverse (RO) oligonucleotides: CWP2-5'FO, 5′-AACTTAAGATATGAACTAAAAAGAAACAG-3′; CWP2-5'RO, 5′-TATTTTCCCAGCCACTGTTGAG-3′; VSP1267-5'FO, 5′-TCCCTCAGCTTATTA TAGGGT A-3′; VSP1267-5'RO, 5′-GCATAGTAATAACCAGATGAATCTTT-3′; VSP9B10-5'FO, 5′-GGTCAACGTGTGCCAGAAGG-3′; VSP9B10-5'RO, 5′-TGCCCGATTGATAGATTGGTAGG-3′; CWP2-Cod.FO, 5′-GTGCTAATTGGAAGTCGAATAACTG-3′; CWP2-Cod.RO, 5′-GAGAGGTCGATCCCAATAACG-3′; VSP1267-Cod.FO, 5′-GGAGTTGCTGGATGTGCTAAAT-3′; VSP1267-Cod.RO, 5′-ACGTTTGTTCATCTGCTTCTGT-3′; VSP9B10-Cod.FO, 5′-AAGACAGCCAAGGACCAAGC-3′; VSP9B10-Cod.RO, 5′-

GGTCTTACAGGTCTTGCATGTTTC-3′.

3. Results

3.1 Histones of Giardia lamblia.

Giardia histones were purified from trophozoites and then separated by SDS-PAGE (**Fig 1A**). *Giardia* histones showed a similar pattern of migration to that of chicken histones. *Giardia* H3 possesses a higher molecular weight (16.3 kDa) compared with the chicken homologue (15.4 kDa). These results agree with the previous report by Triana *et al*. (Triana et al., 2001). By sequence homology, *Giardia* histones have the typical amino acid residues that could be post-translationally modified (**Fig S1**).

3.2 Histone post-translational modifications in Giardia.

In the last years, antibodies that recognize particular histone modifications have been increasingly developed, but they frequently do not cross-react between different species (Villar-Garea and Imhof, 2006). Therefore, we first used Western blotting to test many commercial antibodies on purified *Giardia* histones for the presence of some of the most common histone modifications. Only few antibodies showing specific reactivity for the recognition of euchromatin (H3K4m3, H3K9ac, H4K8ac and H4K16ac), heterochromatin (H3K9m3) and intermediate states (H3K4m1 and H3K4m2) were found useful for subsequent studies. The specificity of these antibodies was determined by competition assays using peptides that contain each specific histone post-translational modification (**Fig 1B**). As a result, these antibodies also certified the identity of the *Giardia* histones preparation. These selected antibodies were then used to determine the specific localization of histone marks during *Giardia* proliferation, encystation and antigenic variation.

3.2.1 Histone H3 lysine 4 methylation.

This is one of the most conserved histone modifications among eukaryotes. H3K4 can be mono- (H3K4m1), di- (H3K4m2) or tri-methylated (H3K4m3), each having different biological functions (Santos-Rosa et al., 2002; Vaquero et al., 2004). These three marks are homogenously distributed in both nuclei of the parasite (**Fig 2A**) and do not undergo major microscopic changes during *Giardia* adaptive processes (not shown).

3.2.2 Histone H3 lysine 9 tri-methylation.

This classical modification is associated with heterochromatin (Guetg and Santoro, 2012) and pre-mRNA splicing (Davie et al., 2016). Results of IFA showed that this mark concentrated in 2-4 spots per nucleus, with a small increase in its intensity during encystation (**Fig 2B**) but not during antigenic variation (**Fig 3**).

3.2.3 Histone H3 lysine 9 acetylation.

This modification is possible when the corresponding lysine is not methylated, and H3K9ac was one of the first histone modifications related to active genes (Santos-Rosa et al., 2002; Villar-Garea and Imhof, 2006). In all our observations, immunolabeling of H3K9ac showed a weak signal in trophozoites (**Fig. S2**).

3.2.4 Histone H4 acetylation.

Antibodies that recognize the four acetylated lysines of histone H4 that identify euchromatin (K5, K8, K12, and K16) have been used in chromatin studies in many eukaryotes (Santos-Rosa et al., 2002; Villar-Garea and Imhof, 2006). Here, we used antibodies specific for two of these modifications (K8 and K16) to determine any potential adaptive differences in the levels of modification. The signal shown by these antibodies was distributed almost homogeneously in both nuclei of *Giardia* trophozoites (not shown). Interestingly, the intensity of H4K8ac and H4K16ac staining decreased during the first hours of encystation, returning to the initial levels after 4-5 h, as compared to vegetative trophozoites (**Fig 4**). By contrast, under the

stimulus for antigenic variation, only acetylation of H4K8 showed an increase in intensity and a change in localization from mostly homogeneous to a peripheral distribution in both nuclei of the parasite (**Fig 3**).

In all cases, visual observations correlated with digital quantification of the signals (**Fig. S3**).

3.3 Histone Deacetylases in Giardia.

Only one homologue of the NAD^+-independent deacetylase (HDAC) and five of the NAD^+-dependent group (Sirtuins) were identified by homology searches. These homologues are the same as those previously identified by Sonda *et al*. (Sonda et al., 2010). However, neither localization nor individual participation of these enzymes during *Giardia* differentiation was previously reported.

3.3.1 NAD+-independent deacetylase - ORF3281 or gHDAC.

The gHDAC belongs to class I of the general classification of the HDACs (Yang and Seto, 2008) (**Fig S4** and **Fig 5A**). This enzyme possesses an N-terminal catalytic domain with high homology to other known enzymes (*Sacharomyces* Rpd3, human HDAC 1-2-3). The primary sequence shows residues that can interact with Zn^{2+}, necessary for catalytic function. The typical localization of these homologues is nuclear in other organisms (de Ruijter et al., 2003), but gHDAC lacks either typical nuclear localization signals (NLS) or nuclear import motifs. To localize this enzyme and understand its function, ORF3281 was epitope-tagged and over-expressed in trophozoites. Western blotting showed one band of ~54 kDa, in agreement with its predicted molecular mass (**Fig 5B**). By IFA, the distribution of gHDAC was mostly nuclear in proliferating trophozoites and perinuclear in cyst (**Fig 5C**). Interestingly, during encystation this enzyme localized in a punctuate pattern in encysting trophozoites and, in some cases, the mark co-localized with that of the Encystation-specific Secretory Vesicles (ESVs), which transport cyst

wall proteins for subsequent release and assembly into the protective cyst wall (Lujan et al., 1997).

3.3.2 NAD$^+$-dependent deacetylases - Sirtuins of G. lamblia.

Giardia has five ORFs with Sir2-like domains. These ORFs present the necessary domains for the catalytic activity and the binding of NAD^+. Mutations of the HG domain generate the loss of deacetylase and ADP-ribosylation activity (Frye, 2000); however, all the *Giardia* homologues maintain these amino acid residues (**Fig S5**). Based on the classification of Frye (Frye, 2000), ORF10707, ORF10708 and ORF11676 belong to Class I. ORF16569 shows homology with Class III Sirt5 from humans, whereas ORF6942 presents low homology with Class I to IV Sirtuins, suggesting it could belong to the subclass U (**Fig 6A**). All the *Giardia* Sirtuins were expressed with C-terminal epitope-tags in trophozoites and their molecular weight and subcellular localization evaluated by immunoblotting (**Fig 6B**) and IFA (**Fig 6C**).

3.3.2.1 ORF10707 – gSir1.

This Sirtuin has an estimated mass of ~63 kDa. The primary sequence has a monopartite and a bipartite NLS. This Sirtuin has high homology with the human SIRT1, which is one of the most widely studied histone deacetylases (Frye, 2000). The distribution of this enzyme is at the periphery of both nuclei (**Fig 6C**), which coincides with the localization of the H4K8 mark during antigenic variation (**Fig 3**).

3.3.2.2 ORF10708 – gSir2.

This is the largest Sirtuin of *Giardia*. Its molecular weight, ~74.6 kDa, agrees with the detection of the ORF10708-HA construct by immunoblotting (**Fig 6B**). The cellular localization is cytoplasmic; therefore, the function of this protein could not be related to gene regulation and chromatin structure (Vaquero, 2009).

3.3.2.3 ORF16569 – gSir3.

It is the smallest Sir2-like protein of *Giardia*. Its subcellular distribution is at the periphery of the nuclei (**Fig 6C**). This sequence is more closely related to the Sir5 of humans and belongs to type III (**Fig 6A**). Class III Sirtuins are the most widely distributed and are found from bacteria to humans (Vaquero, 2009).

3.3.2.4 ORF6942 – gSir4.

This ORF has an estimated molecular weight of 73 kDa. Despite the size of this putative Sirtuin, it cannot be grouped into Fyre´s classification (Frye, 2000) probably due to the small and incomplete Sir2-like domain. This protein has two monopartite NLS, ensuring its nuclear localization (**S5 Fig** and **Fig 6A**).

3.3.2.5 ORF11676 – gSir5.

This homologue possesses a molecular mass of 66.5 kDa and has a putative monopartite NLS. Interestingly, this enzyme is distributed in two or three spots in both nuclei of the parasite, similarly to the H3K9m3 pattern (**Fig 2B**). Only one nuclear domain was previously identified in *Giardia*: the nucleolus (Jimenez-Garcia et al., 2008). This structure is associated with heterochromatin in other organisms (Guetg and Santoro). Thus, an antibody that labels the nucleolus, anti-2, 2, 7-methyl guanosine (Meskauskas et al., 2003), was used for comparison. This antibody identified just one polar domain that did not merge with the H3K9m3 spots (**Fig S6**). The comparison of the relationship between the distribution of gSir5 and the H3K9m3 marks shows a partial colocalization of both marks. In addition, we observed a partial overlap of gSir5 and the nucleolus of *Giardia* (**Fig S6**), suggesting that this deacetylase could be involved in ribosomal RNA processing (Meskauskas et al., 2003).

3.4 Histone Deacetylases and Giardia Differentiation.

Based on the changes observed at the level of the acetylation of histones during encystation and antigenic switching, we decided to focus our work on the enzymes that deacetylate histones during *G. lamblia* differentiation processes.

3.4.1 Treatment with histone deacetylase inhibitors during the encystation process.

Trichostatin A (TSA, a hydroxaminic acid derived from *Streptomyces*) and Sodium Butyrate (NaB, a short-chain lipid acid), which inhibit the NAD^+-independent deacetylases by blocking the enzyme active site, and Nicotinamide (Nt), an inhibitor of the NAD^+-dependent deacetylases (Xu et al., 2007), were used. Because some of these enzymes are known to be important for normal cell functioning, initial experiments (not shown) determined the inhibitor concentrations that did not affect trophozoite growth: TSA 200 nM, NaB 20 nM and, Nt 10 mM. To prove the influence of the histone deacetylases on the encystation process, we exposed trophozoites to encystation medium at different concentration of the inhibitors. After 24 h in the presence of the inhibitor, cells were stained using a mAb against a CWP, and the number of cysts released to the culture medium was quantified. Encystation was blocked in the presence of the three inhibitors (**Fig 7A**). To determine if inhibition was due to transcription disruption or cyst wall component transport, trophozoites were induced to encyst for 1, 2, 4, 8, 12 and 24 h in the presence or absence of the inhibitors, then lysed and used in immunoblotting assays with an anti-CWP1 mAb. **Fig 7B** shows that inhibition of encystation is due to inhibition of expression of encystation-specific molecules and not to alteration of intracellular transport.

Since changes in the acetylated lysine patterns (H4K8ac and H4K16ac) were observed during the first hour of encystation (**Fig 2**), we replicated this experiments but adding the inhibitors 4 h after the start of encystation. In the latter experiment, formation of mature cysts and expression of CWPs were observed (**Fig 7C**). Taken together, these results indicate that the first

stage of encystation is in part regulated by the activity of both NAD^+-dependent and NAD^+-independent histone deacetylases, and not only by the gHDAC, as reported (Sonda et al., 2010).

3.4.2 Treatment with histone deacetylases inhibitors during antigenic variation.

To evaluate a putative link between histone deacetylases and antigenic variation, clonal population of trophozoites expressing a particular VSP were obtained (VSP9B10, VSP1267 and VSPA6). The expression of a given VSP was confirmed using specific anti-VSP mAbs and switching over time was followed by IFA (**Fig 8A**). These homogeneous populations were divided into different tubes and exposed to TSA, NaB or Nt. After periods of 48 h, the culture medium containing fresh inhibitors was replaced. Results showed a rapid and clear increase in the switching rate of the original VSPs in the presence of the TSA and minor effects in the presence NaB or Nt, all compared to the controls without inhibitors. At day 5, a strong drop in the expression of the original VSP was observed in the presence of TSA, and less strong with NaB and Nt. The rate of switching was similar, independently of the initial VSPs expressed in each clone. In these experiments, antigenic switching was verified by using antibodies to different VSPs. Given the possibility of *Giardia* VSPs to switch to one among ~200 different variants, the limitation of available anti-VSP mAbs allowed the identification of the newly-expressed VSP only in few cases (not shown). Nevertheless, this particular observation confirmed that inhibitor treatment promotes antigenic variation rather than VSP surface disappearance by reducing its expression.

Similar results were observed using quantitative PCR (**Fig 7B).** The VSP1267 mRNA level (from a clonal population only expressing VSP1267 on its surface) was found at higher concentration, compared with other VSP transcripts that are not expressed on the parasite surface (VSP9B10 and VSPA6). In the presence of TSA, the expression of the VSP1267 increased rapidly during the first day of growth but decreased by day 5, although the expression of this

surface antigen was observed by IFA in a high number of cells during this period (**Fig 8A**). Nicotinamide decreased the expression of VSP1267 mRNA, but it remained at high concentration at the protein level when compared with other VSPs.

3.4.3 Putative histone deacetylases targets.

The deacetylases have several substrates, including histones (de Ruijter et al., 2003; Vaquero, 2009). Therefore, the changes that inhibitors produce in the profile of histone modification of *Giardia* were evaluated during trophozoite differentiation into cysts. Trophozoites cultured in encystation medium were exposed to the different inhibitors, and IFAs with different anti-histone modification antibodies were subsequently performed. This showed an increase in the signal of H3K9ac and H4K8ac when the cells were treated with TSA and NaB, indicating that NAD^+-independent histone deacetylases affect these modifications. Nicotinamide increased the level of H3K9ac, H4K8ac and, remarkably, H4K16ac (**Fig S7**).

3.4.5 Chromatin immunoprecipitation.

To gain deeper insights into the association between different histone modifications and specific genes related to the *G. lamblia* differentiation processes, chromatin immunoprecipitation assays (ChIP), followed by amplification and quantification by PCR, was performed (**Fig 9**). Two regions of each gene were chosen to design primer sets: one pair to include the 100 bp segment upstream of the start codon and the other to include the coding region. The CWP2 gene, which is known to be up-regulated more than 170 times during encystation (Lujan et al., 1997), and the VSP1267, VSPA6 and VSP9B10 genes were selected to study both encystation and antigenic variation. For encystation, the starting material were nuclei extracted from trophozoites grown in culture medium without inhibitors and trophozoites grown in encystation medium for 1-24 h, with and without histone deacetylases inhibitors (TSA and Nt). For antigenic variation, the material was isolated from clonal populations that only express VSP1267 grown in culture

medium for 24 h, with or without the presence of histone deacetylase inhibitors (TSA and Nt).

Regarding encystation, we observed a decrease in the interaction of H4K8ac with the 5' upstream region of the CWP2 gene during the first hours of culture in encystation media (**Fig 9A**); this result is in agreement with the decrease of the H4K8ac levels observed by IFA (**Fig 2A**). This decrease was not significant for the coding region. In encysting cells (24 h in encystation medium), this interaction was the same as that of the 5´upsteam region of H4K8ac, as well as for H3K9ac and H4K16ac. These results contradict the general idea that acetylation is associated with gene expression; however, at this stage of differentiation, the trophozoites progress into a dormant state where gene expression in general is down-regulated, except for the genes necessary for transport and assembly of the cyst wall constituents (Lujan et al., 1997). A decrease in H3K4m3 at the 5' upstream region was also observed, reinforcing this hypothesis. In the presence of TSA, the level of H4K8ac was higher at the 5´upstream and coding regions. H4K16ac is apparently not related to the 5' upstream or coding region of CWP2. Interestingly, the presence of TSA decreases the interaction of the 5' upstream region of CWP2 with H3K4m3, showing that the methylation level of lysines is also involved in the encystation process.

Concerning the results of ChIP assays for the VSP genes, in a clonal population the 5' upstream region of VSP1267 was highly associated with H4K8ac (and not with H3K9ac or H4K16ac), compared with other VSPs that are not expressed. In the presence of TSA (24 h), a significant increase of the association of VSP1267 5' upstream region with H3K9ac, H4K8ac and H4K16ac was observed (**Fig 9B**). These results agree with the increase in the level of histone modification marks and VSP expression (**Fig 3** and **Fig 9**). No significant changes were found in the coding region of VSP1267. In the presence of Nt, only an increase in the association of H4K16ac with the 5' upstream region of VSP1267 was detected. When genes that are not expressed (VSP9B10 and VSPA6) were analyzed, a significant decrease in the association of the

acetylation marks with the 5' upstream and coding region was also observed. These results suggest that H4K8ac is the most important histone modification associated with the VSP that is expressed, and H3K9ac and H4K16ac may collaborate in this process by increasing the transcription rate. Remarkably, the changes found in the presence of TSA correlated with an increase in H3K9m3 at the 5' upstream region of the VSP1267. These results indicate that the actual VSP increases its expression during the initial moments of inhibitor treatment but is then silenced, with the H3K9m3 modification being involved in this process in a direct manner.

3.4.4 Effects of histone deacetylase over-expression on Giardia differentiation.

All the histone deacetylases encoded in the *Giardia* genome were expressed in trophozoites together with a C-terminal HA epitope tag to determine their subcellular localization (**Fig. 4-6**). In these populations, we also compared the rate of *Giardia* differentiation between wild type trophozoites and those expressing different histone deacetylases under the control of the α-tubulin upstream region. Results indicate that that over-expression of HDAC and Sir5 shows a slight increase in the level of trophozoite differentiation into cysts and that over-expression of HDAC, Sir3, and Sir5 reduces the rate of antigenic variation, more likely by decreasing the levels of acetylation of histone H4K9 and H3K8 and H4K16, as determined by the immunofluorescence and ChIP assays.

4. Discussion

The binucleate protozoan *G. lamblia* presents the four classic core histones, although a typical linker histone H1 is missing in its genome. Here we identified, for the first time in this early-branching protist, some of the best known modifications associated with euchromatin, heterochromatin, and intermediate conditions and described their mechanistic relationship to *Giardia* encystation and antigenic variation.

4.1 Histone Modifications and Encystation.

When *Giardia* trophozoites sense the stimulus for encystation, the expression of encystation-specific genes is upregulated (Lujan et al., 1997). Here we describe that *Giardia* encystation is partly controlled by a histone deacetylation event that takes place very early after the trophozoites are exposed to the stimulus for encystation. At this stage, the general level of H4K8ac and H4K16ac decreases. We also found a high basal level of H4K8ac associated with the 5´upstream region of an encystation-specific gene in non-encysting trophozoites, when this gene is not expressed (Lujan et al., 1997), suggesting that this open chromatin state may be necessary for repression of encystation-specific gene expression. Conversely, a significant, but not complete, decrease in the association of the H4K8ac mark with the ´5 upstream region of CWP2 gene occurs early during encystation. Later in the process, when the cysts are being formed, the global level of acetylation returns to normal and the association with the 5' upstream region of the CWP2 gene remains low. H4K8ac alone has been scarcely described in other organisms, especially in early-branching protozoan parasites. However, a recent report showed that variations in the level of H4K8ac in *Plasmodium falciparum* regulate transcription at different stages of its life cycle (Chaal et al., 2010). In *Giardia*, similarly, this histone mark may be involved in the fine-tuning of transcriptional control, since most of the regulation of gene expression seems to occur post-transcriptionally (Prucca et al., 2008).

Our results suggest that the activity of a NAD^+-independent deacetylase (gHDAC), and NAD^+-dependent deacetylases (gSirtuins) are important during encystation. These results partially agree with a previous report from Sonda and coworkers (Sonda et al., 2010), who reported the inhibition of encystation by a different NAD^+-independent deacetylase inhibitor. These authors were unable to neither specify when this histone deacetylation activity is necessary for encystation nor characterize specific substrates for these enzymes and the NAD^+-dependent

group of histone deacetylases.

The *Giardia* ORF3281 is the only representative member of the HDAC family. The protein sequence shows the presence of several putative phosphorylation sites that can be modified by Ca^{2+}/calmodulin-dependent kinases. These kinases regulate the HDAC activity, subcellular location and the binding to other elements such as the 14-3-3 protein. *Giardia* has homologues of these proteins, which have been suggested to participate in the regulation of encystation (Alvarado and Wasserman, 2011; Lalle et al., 2012). The HDACs have been widely related with gene regulation and stage differentiation in protozoa and other organisms (Bougdour et al., 2009; Chaal et al., 2010; Ingram and Horn, 2002). In our experimental conditions, when the activity of gHDAC was inhibited during encystation, an increase in the signal of H3K9ac, H4K8ac and, to a lesser degree, of H4K16ac was observed. At the 5' upstream region of the encystation-specific CWP2 gene, we found a persistent association with H4K8ac and H4K16ac and a poor association with H3K9ac. Interestingly, the level of H3K4m3 decreased at the upstream region of the CWP2 gene, suggesting that the degree of acetylation clearly depends on the level of histone methylation.

Sir2-like enzymes are intrinsically associated with chromatin regulation and gene silencing and, interestingly, they may function as metabolic sensors. They have specificity to deacetylate H4K16ac and, to a lesser extent, H3K9ac (Fulco et al., 2003). Among the four H4 lysines, K16 is the most frequently acetylated and it has been associated with euchromatin from yeast to humans (Vaquero et al., 2007). In addition, acetylation of H4K16 is associated with the disruption of the formation of the 30-nm fiber and, consequently, of heterochromatin. Studies in yeast associate the formation of heterochromatin with deacetylation and the activity of Sirtuin at two conserved cellular localizations: telomeres and the nucleolus (see below, section 4.3).

In the presence of acetylated proteins, Sirtuins can metabolize NAD^+ into nicotinamide

and ADP-ribose. Some of these enzymes can work like mono-ADP-ribosyltransferases, but the presence of this modification has still not been described in *Giardia.* The inhibition of these enzymes increases the level of H4K8ac, H4K16ac and H3K9ac. However, there is a decrease of the association of H4K8ac and H4K16ac at the CWP2 gene 5' upstream region. As a general concept, deacetylation of histones is likely related to CWPs gene expression.

Several factors have been described to be involved in *Giardia* encystation (Gillin et al., 1989), but cholesterol deprivation was clearly demonstrated to be necessary and sufficient to trigger this process (Lujan et al., 1996). When *Giardia* trophozoites travel down the intestine, the parasites sense a low lipid concentration and start encysting (Carranza and Lujan, 2010; Lujan et al., 1996). The ileum is not only a low-cholesterol environment; it is, in fact, a region poor in many nutrients, especially those that *Giardia* is unable to synthesize (Jarroll et al., 1989). This nutritional restriction might make *Giardia* to adapt to a more efficient use of energy (Vaquero and Reinberg, 2009). This hypoenergetic condition could increase the level of NAD^+, activating different pathways such those including Sirtuins and AMP-dependent kinases (Hardie et al., 2006). The cytoplasmic [NADH]/[NAD^+] ratio can influence gene expression and, therefore, the differentiation process by the histone deacetylation activity of the Sirtuins.

Our results and those of Sonda *et al*. (Sonda et al., 2010) suggest a mechanistic model in which the involvement of a constitutively expressed repressor of encystation may play an essential role. The steady-state level of histone acetylation may allow repressor expression and, consequently, the impairment of encystation under normal *Giardia* proliferation.

4.2 Histone Modifications and Antigenic Variation.

The activity of the only *Giardia* homologue of HDAC is involved in the switching of one VSP for another from a repertoire of ~200 different variants. Kulakova *et al*. (2006) first suggested that epigenetic mechanisms would be involved in the control of antigenic variation in

Giardia. These authors integrated a DNA fragment comprising the VSPH7 gene fused to the HA epitope (VSPH7-HA), all surrounded by the VSPH7 5' and 3´ gene flanking sequences, into the genome of parasites of the GS isolate, which usually express VSPH7 on their surface. Surprisingly, these cells expressed on their surface either the native VSPH7 or the integrated version, indicating that the expression of these genes is independent of each other despite having almost the same sequences. These authors also found that acetylation of histones in the 5' flanking region of VSPH7-HA favored its expression. They proposed that epigenetic mechanisms were involved in VSP gene transcription activation, which was later refuted since most of VSPs are simultaneously transcribed (Prucca et al., 2008). The fact that one VSP transcript evades degradation by RNAi is likely due to differences in VSP transcription efficiency mediated by specific histone tail modifications. Accordingly, the cross-talk between histone modifications and the RNAi pathway has been clearly documented (Goto and Nakayama, 2012).

The acetylation/deacetylation profile of histones is involved in the regulation of the antigenic variation of two other important protozoan parasites, *Plasmodium falciparum* and *Trypanosome brucei*. An important difference from *Giardia* is that these organisms regulate this process at the transcriptional level, where the chromatin structure plays a key role. The regulation of the expression of the telomeric VSG genes (Variable Surface Glycoproteins) of *T. brucei* takes place in nuclear domains, where a VSG gene is transcribed by RNA polymerase I (Figueiredo et al., 2008; Wang et al., 2010; Yang et al., 2009). In *P. falciparum* the *var* genes that are not expressed are located in telomeric heterochromatin regions and, to be expressed, they need to relocate to subnuclear activation domains, where a PfSIR2 is involved (Bougdour et al., 2009; Chaal et al., 2010; Scherf et al., 2008). It is possible that the nuclear domains labeled by the H3K9m3 mark described here participate in the regulation of the VSP expression.

4.3 Heterochromatin in *Giardia*.

Heterochromatin has been traditionally characterized by three biochemical marks: hypoacetylation of histones, H3K9 methylation and DNA methylation (Guetg and Santoro, 2012). In this work, we identified the presence of heterochromatin in *Giardia* for the first time. This histone modification is concentrated in 2-4 nuclear domains that increase slightly during encystation. These dots partially colocalize with the *Giardia* Sir2-like homologue ORF11676, but this phenomenon is not associated with silencing of repetitive ribosomal DNA sequences (Meskauskas et al., 2003). On the other hand, *Giardia* lacks other conserved components that participate in the formation of heterochromatin, such as the heterochromatin protein 1 (HP1) and histone H1. These results could explain why some reports indicate that *Giardia* has a relaxed mechanism controlling transcription, allowing the simultaneous expression of different VSP genes, although with variable efficiency (Prucca et al., 2011).

In summary, we here report for the first time how specific histone modifications participate in the control of gene expression during *Giardia* adaptation to different environmental conditions. Our results pave the way for future studies regarding the complexity of the gene regulatory machinery in this important human pathogen.

Conflicts of interest

All authors have read the journal's policy on disclosure of potential conflicts of interest and they have none to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version and includes:

Fig. S1. Amino acid sequence alignment of *Giardia* histones with those from other organisms.

Fig. S2. Localization of the histone H3 lysine 9 acetylation.

Fig. S3. Quantification of signals of different histone modifications.

Fig. S4. Amino acid sequence alignment of Class I histone deacetylases.

Fig. S5. Amino acid sequence alignment of Class III histone deacetylases.

Fig. S6. Heterochromatin in *Giardia* nuclei.

Fig. S7. Histone acetylation in the presence of deacetylase inhibitors.

Fig. S8. Influence of over-expression of different histone deacetylases on *Giardia* differentiation.

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Figure Legends

Fig 1. Histones and histone modifications in *G. lamblia*. A. The quality of purified histones containing histone H3, H4, H2A/H2B was determined by SDS-PAGE and Coomassie blue staining, and compared with purified Chicken histones. B. Peptide competition assay demonstrates the specificity of the selected antibodies against H3 and H4 modifications. The molecular weights are indicated on the left of each figure.

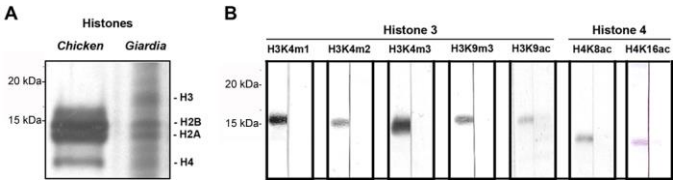

Fig 2. Localization of histone methylation marks during *Giardia* encystation. IFAs were carried out with antibodies against specific H3 and H4 lysine residue methylation (green) in proliferating trophozoites, encysting trophozoites and cysts. A. H3K4m1, H3K4m2 and H3K4m3. B. H3K9m3. In B, the encystation process was confirmed using a specific mAb against CWP2 (red). Nuclear DNA was stained with DAPI (blue). White bar represents 2.5 µm for all figures.

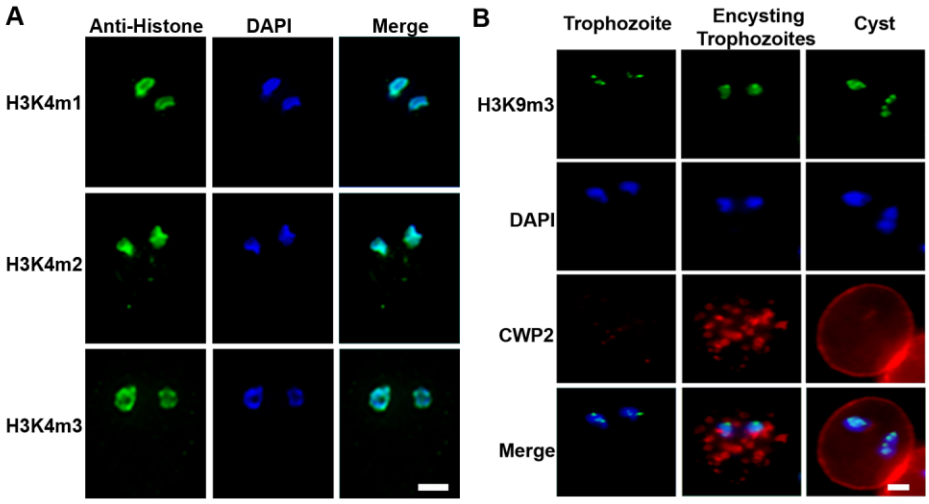

Fig 3. Localization of specific histone marks during *Giardia* antigenic variation. Trophozoites expressing a particular VSP were incubated with a specific mAb against this VSP at different times after induction of antigenic variation. IFAs were carried out with antibodies against specific H3 and H4 lysine residue methylation and acetylation (green). VSP was detected using a mAb against VSP9B10 (red). The inset shows the perinuclear localization for H4K8ac and the nuclear DNA was stained with DAPI (blue). White bar represents 5 μm for all figures.

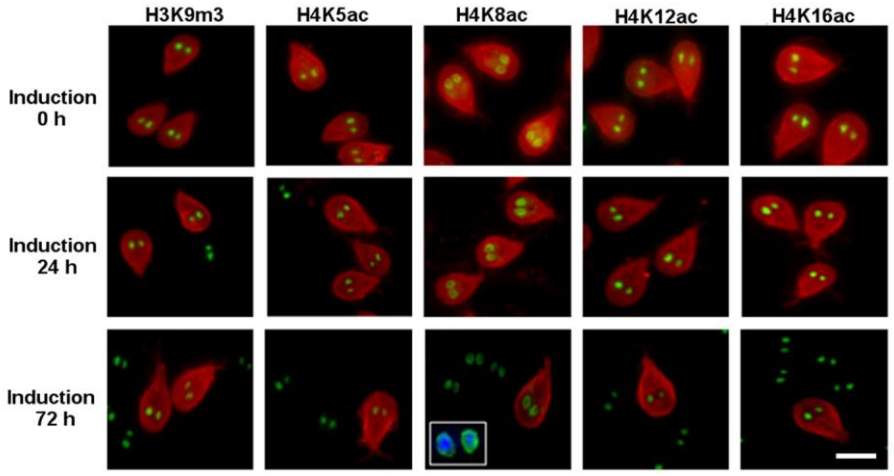

Fig 4. Localization of histone acetylation during *Giardia* encystation. IFAs were carried out with antibodies against H4K8ac or H4K16ac (green) in proliferating trophozoites, encysting trophozoites and cysts. The encystation process was confirmed using a specific mAb against CWP2 (red). Nuclear DNA was stained with DAPI (blue). White bar represents 5 µm for all figures.

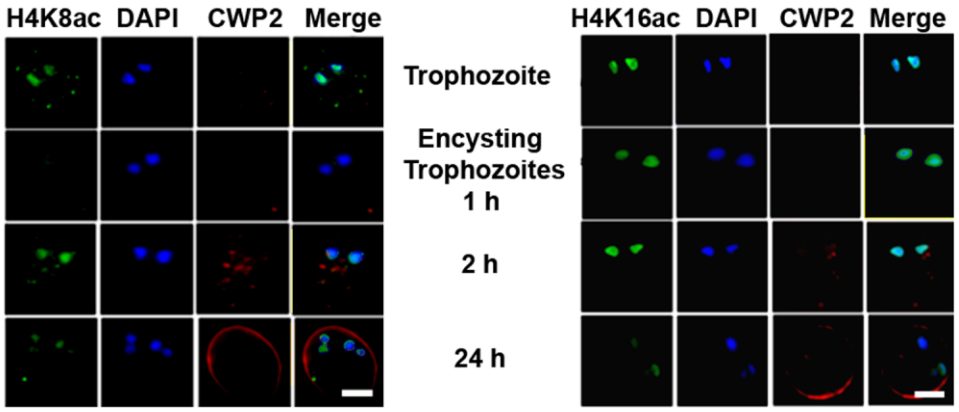

Fig 5. *Giardia* Class III histone deacetylases. A. Phylogram showing relationships among the ORF3281 and 11 human (Hs) histone deacetylases. The phylogram was generated from multiple sequence alignment of the entire amino acid sequence from each HDAC. B. Western blot from transfected trophozoite protein extracts using an antibody against the HA tag. C. Immunofluorescence assays of transfected trophozoites expressing the histone deacetylase ORF3281 coupled to a HA-tag, grown in normal and encysting media. A specific mAb was used directed to the HA-tag (red) and the encystation process was confirmed with a specific mAb against CWP1 (green). Nuclear DNA was stained with DAPI (blue). White bar represents 5 µm for all figures.

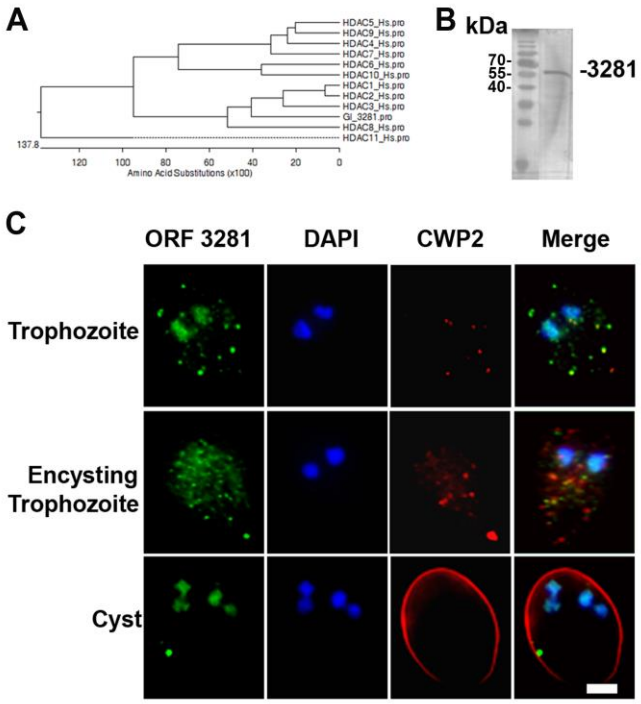

Fig 6. *Giardia* Class III histone deacetylases. A. Phylogram showing relationships among the seven human (Hs) Sirtuins and the five *Giardia* ones. The phylogram was generated from multiple sequence alignment of the SIRT domain amino acid sequence from each Sirtuin. B. Western blot from transfected trophozoites protein extracts using an antibody against the HA tag. The molecular weight is indicated in each arrow. C. Immunofluorescence assays of transfected trophozoites expressing the histone deacetylases ORF6942, ORF10707, ORF16569 and ORF10708 coupled to a HA-tag, grown in normal medium. A specific mAb was used directed to the HA-tag (red) and the nuclear DNA was stained with DAPI (blue). White bar represents 5 µm for all figures.

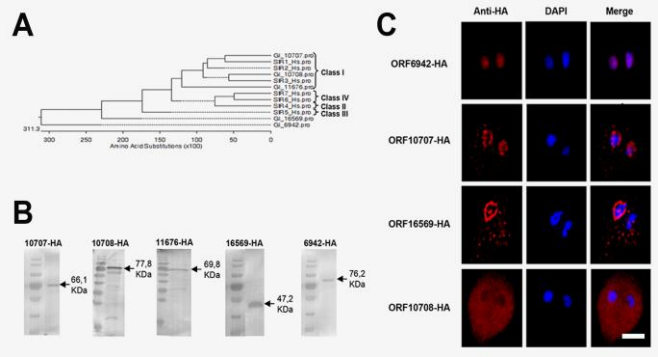

Fig 7. Effects of histone deacetylase inhibitors on *Giardia* encystation. A. Graphs depicting the number of cysts formed per milliliter of encystation medium under increasing concentrations of deacetylase inhibitors. The figures represent the mean value of three independent experiments ± SD. B. Western blots of trophozoite growth in normal medium (N) and in encystation medium for different periods (1, 2, 4, 8, 12 and 24 h) in the presence or absence of the indicated deacetylase inhibitor. C. Similar experiments in which the deacetylase inhibitors were added after 4 h of the start of encystation. A specific mAb against CWP1 was used to follow the encystation process.

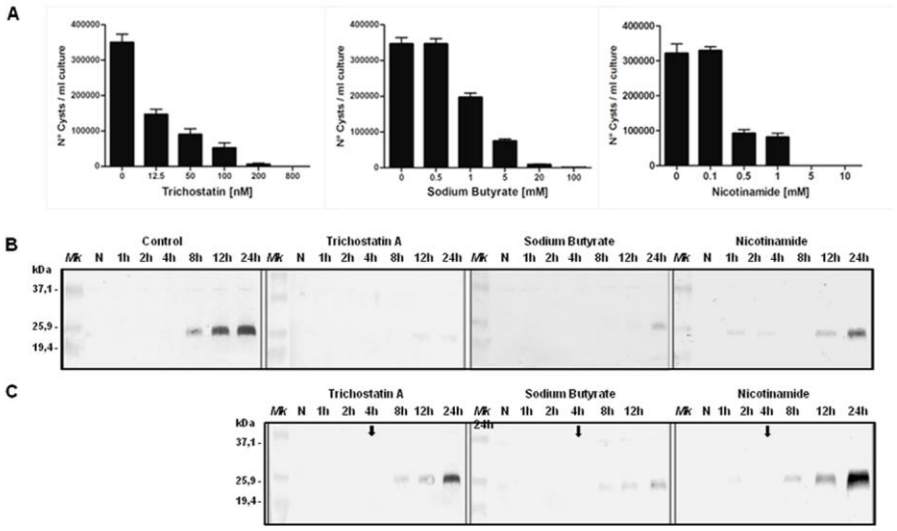

Fig 8. Effects of histone deacetylase inhibitors on *Giardia* antigenic variation. Clonal trophozoite populations were grown in the presence of histone deacetylase inhibitors. A. Cultures were monitored by IFA using specific mAb against VSP9B10 (green) for 5, 12 and 23 days. Nuclear DNA was stained with DAPI (blue). White bar represents 100 µm for all figures. B. The relative expression of three VSP transcripts was determined by qPCR during the first and fifth day after the inhibitor was added to the culture medium. The figures represent the mean value of three independent experiments ± SD.

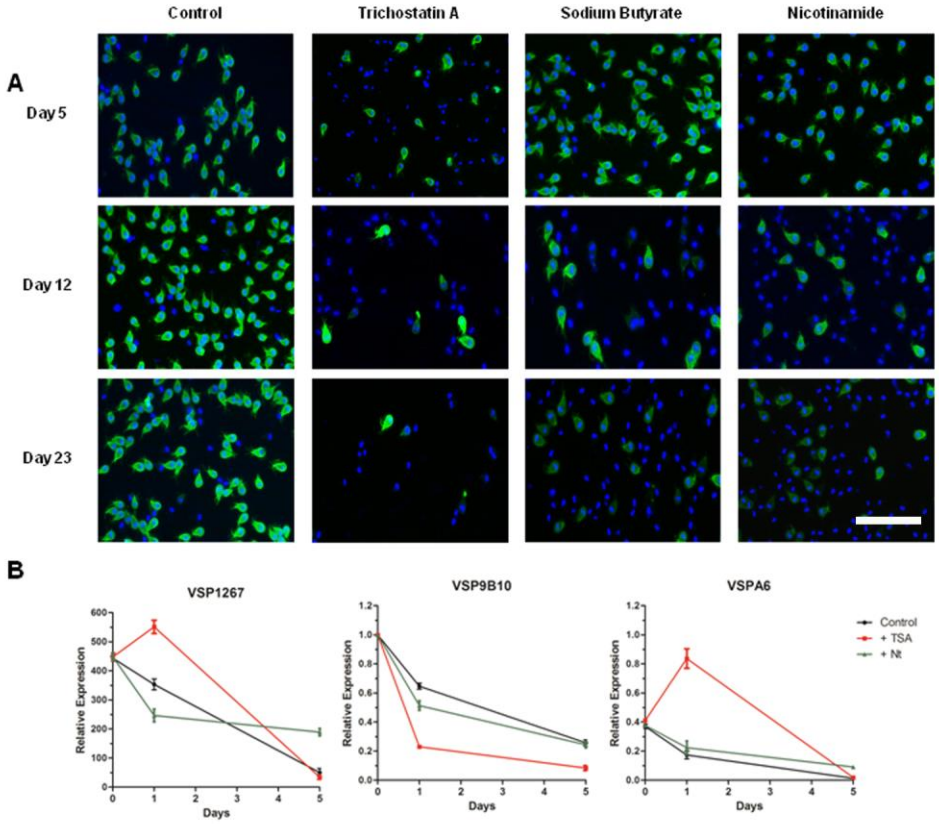

Fig 9. Chromatin Immunoprecipitation assays. ChIP was performed using mAbs against different histone modifications and quantified by qPCR using specific primers from the 5´-UTR or the coding sequence of genes related to encystation and antigenic variation. A. Trophozoites were grown in normal or encystation media for 1 h (1E) or 24 h (24E) without inhibitors. Trophozoites were grown in encystation media for 24 h with histone deacetylase inhibitors (TSA or Nt). Primers for qPCR were designed to target the 100-bp segment upstream of the start codon (5´-UTR) or to the coding region (cod) of CWP2 gene. B. A clonal population of trophozoites expressing the VSP1267 was grown in normal medium for 24 h with or without the presence of TSA or Nt. The primers for qPCR were designed to target the 100-bp segment upstream of the start codon (5´-UTR) or to the coding region (cod) of VSP1267 and the VSP9B10 genes. The figures represent the mean value of three independent experiments ± SD.