# EPIGENETIC REGULATES TRANSCRIPTION AND PATHOGENESIS IN THE PARASITE *TRICHOMONAS VAGINALIS*

***Tomas Pachano [1]; Yesica R. Nievas [1]; Ayelen Lizarraga [1]; Patricia J. Johnson [2]; Pablo H. Strobl-Mazzulla [3]; Natalia de Miguel [1]****

*1 Laboratorio de Parásitos Anaerobios, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico Chascomús (IIB-INTECH), CONICET-UNSAM, Chascomús B7130IWA, Argentina*

*2 Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, California 90095-1489, USA*

*3 Laboratorio de Biología del Desarrollo, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico Chascomús (IIB-INTECH), CONICET-UNSAM, Chascomús B7130IWA, Argentina*

*Corresponding author. Email: ndemiguel@intech.gov.ar (NdM)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cmi.12716

**SUMMARY**

*Trichomonas vaginalis* is a common sexually transmitted parasite that colonizes the human urogenital tract. Infections range from asymptomatic to highly inflammatory, depending on the host and the parasite strain. Different *T. vaginalis* strains vary greatly in their adherence and cytolysis capacities. These phenotypic differences might be attributed to differentially expressed genes as a consequence of extra-genetic variation, such as epigenetic modifications. In this study we explored the role of histone acetylation in regulating gene transcription and pathogenesis in *T. vaginalis*. Here, we show that histone 3 lysine acetylation (H3KAc) is enriched in nucleosomes positioned around the transcription start site of active genes (BAP1 and BAP2) in a highly-adherent parasite strain; compared with the low acetylation abundance in contrast to that observed in a less-adherent strain that expresses these genes at low levels. Additionally, exposition of less-adherent strain with a specific histone deacetylases inhibitor, trichostatin A (TSA), upregulated the transcription of BAP1 and BAP2 genes in concomitance with an increase in H3KAc abundance and chromatin accessibility around their transcription start sites. Moreover, we demonstrated that the binding of IBP39, the transcription factor responsible for the initiation of transcription of ~75% of known *T. vaginalis* genes, depends on the histone acetylation state around the metazoan-like initiator (Inr) to which IBP39 binds. Finally, we found that TSA treatment increased parasite aggregation and adherence to host cells. Our data demonstrated for the first time that H3KAc is a permissive histone modification that functions to mediate both transcription and pathogenesis of the parasite *T. vaginalis*

## INTRODUCTION

*Trichomonas vaginalis* is the flagellated protozoan parasite etiologic agent of trichomoniasis, the most frequent non-viral sexually transmitted infection. Asymptomatic infection by *T. vaginalis* is common, however, numerous symptoms and pathologies occur in both men and women, including vaginitis, urethritis, prostatitis, low birth weight infants and preterm delivery, premature rupture of membranes and infertility (Swygard *et al.*, 2004; Fichorova, 2009). *T. vaginalis* has also emerged as an important cofactor in amplifying human immunodeficiency virus spread (McClelland *et al.*, 2007; Van Der Pol *et al.*, 2008) and increasing the risk of cervical and prostate cancers (Gander *et al.*, 2009; Stark *et al.*, 2009; Sutcliffe *et al.*, 2009; Twu *et al.*, 2014).

The genome of *T. vaginalis* is remarkable; it is the biggest protist genome currently sequenced (estimated to reach up to 175 Mbp in length), encodes ~46,000 protein-encoding genes, is composed primarily of repetitive sequences (up to 65% of the genome) and many gene families are highly expanded (Carlton *et al.*, 2007; Kusdian and Gould, 2014). Transcriptome information currently available reflects the complexity of this genome (Huang *et al.*, 2012; Gould *et al.*, 2013). Only about half of the protein-coding genes appear be expressed (Gould *et al.*, 2013) and individual members of gene families are differently regulated upon environmental changes or within different parasite strains (de Miguel *et al.*, 2010; Horvathova *et al.*, 2012; Gould *et al.*, 2013; Huang *et al.*, 2014; Kusdian and Gould, 2014). These results indicate that extrinsic and intrinsic regulation accurately controls the fine-tuning of gene expression in this extracellular parasite.

Transcriptional regulation governs gene expression, and consequently is a key factor defining the fate of individual cells. Early studies in the field of transcriptional control have been focused on the identification of important players that influence the expression of certain

genes, which led to the discovery of transcription factors and their correlate DNA elements, such as promoters and enhancers. Specifically in *T. vaginalis*, the majority of gene expression studies have been focused on the promoter region of protein-coding genes (Quon *et al.*, 1994; Liston *et al.*, 1999; Liston and Johnson, 1999; Hsu *et al.*, 2009; Smith and Johnson, 2011; Smith *et al.*, 2011). Analysis of the genome predicted that 75% of protein coding genes were found to be surrounded by a metazoan-like Initiator (Inr) element, highlighting the central role this regulatory element plays in *T. vaginalis* gene expression (Carlton *et al.*, 2007). A novel initiator binding protein, IBP39, which interacts with the RNAPII CTD subunit (Schumacher *et al.*, 2003), was identified and demonstrated to recognize this Inr element (Liston *et al.*, 2001). With the exception of the Inr, bioinformatics analyses indicate that *T. vaginalis* does not utilize other previously described eukaryotic core promoter elements (CPEs) to direct transcription initiation (Smith and Johnson, 2011). In summary, the initiation of gene expression in *T. vaginalis* appears to be primarily regulated by a conserved metazoan-like CPE, the Inr element (Liston *et al.*, 1999; Smith *et al.*, 2011).

Although transcription initiation of specific genes directed by CPEs enables basal transcription, levels of gene expression are the consequence of regulatory events. Evidence from other eukaryotes indicates that chromatin structure regulates gene expression through histone modification that alters packaging of DNA and establishes obstacles to transcriptional machinery to operate (Kouzarides, 2007; Shahbazian and Grunstein, 2007; Schones *et al.*, 2008). Chromatin can be fashioned to be transcriptionally repressive or permissive, dependent in part on posttranslational modifications made to histones, a phenomenon referred to as the histone code (Jenuwein and Allis, 2001; Turner, 2002). Acetylation of histone lysine residues at the N-terminal domain plays an important role in the opening of chromatin structure as it weakens the interaction of DNA, destabilizes nucleosome structure and facilitates binding of complexes to promoter regions (Boeger *et al.*, 2003; Reinke and Hörz,

2003; Shahbazian and Grunstein, 2007). The steady state level of histone acetylation is controlled by the dynamic activities of histone acetylases (HATs) and deacetylases (HDACs) (Katan-Khaykovich and Struhl, 2002), which are specifically recruited to regulatory elements where they influence transcriptional activity (Li *et al.*, 2002). These enzymes are highly conserved from yeast to human. HDACs can be grouped into four classes: Class I, II, II and IV based on their homology to yeast orthologs (de Ruijter *et al.*, 2003; Yang and Seto, 2008). Both HATs and HDACs have delicate functions in many cellular pathways and chemicals that inhibit their activities are currently used to study transcription regulation by the acetylation mark. Although epigenetics has been described as a key player in controlling transcription in other cells, including parasites (Freitas-Junior *et al.*, 2005; Saksouk *et al.*, 2005; Janzen *et al.*, 2006; Sullivan *et al.*, 2006; Ehrenkaufer *et al.*, 2007; Iyer *et al.*, 2008; Tonkin *et al.*, 2009; Chaal *et al.*, 2010; Sonda *et al.*, 2010; Coleman *et al.*, 2014), there is no information about the role of epigenetics in transcriptional regulation and its consequences in the pathogenesis process of *T. vaginalis*.

Despite being of critical importance to the survival of *T. vaginalis*, the mechanisms used to establish an infection and thrive within its host are poorly defined. Different *T. vaginalis* strains are highly variable in their adherence, aggregation and cytolysis capacity (Rojas *et al.*, 2004; Lustig *et al.*, 2013; Coceres *et al.*, 2015), even when they have almost identical amino acid sequences ((Leitsch *et al.*, 2009; de Miguel *et al.*, 2010) and our unpublished data). These differences in the pathogenic phenotype could suggest that the proteins mediating these interactions vary greatly, either in abundance or type between strains (Lustig *et al.*, 2013). We have previously identified several genes differentially expressed in adherent versus non-adherent strains of *T. vaginalis* (de Miguel *et al.*, 2010). Specifically, two hypothetical proteins, BAP1 (TVAG_166850) and BAP2 (TVAG_244130), that were more abundant in highly adherent strains, share 33% similarity on the amino acid level and belong to a family

encoded by ~100 genes in the *T. vaginalis* genome (de Miguel *et al.*, 2010). When either of these proteins was expressed upon transfection of a less-adherent strain, attachment to VECs was increased ~2.2-fold (de Miguel *et al.*, 2010); suggesting a possible function in parasite attachment for these abundant surface proteins. Here, we show that histone 3 lysine acetylation (H3KAc) is enriched in nucleosomes positioned around the transcription start site of active genes BAP1 and BAP2 in a highly-adherent parasite strain; in contrast to that observed in a less-adherent strain that expresses these genes at low levels. Additionally, we demonstrated that histone acetylation is likely responsible, at least in part, for the differential gene expression and strikingly different phenotypes observed between strains. Importantly, we revealed, for the first time, that epigenetics plays a central role in regulation of gene expression and contributes to *T. vaginalis* pathogenesis.

## RESULTS

### H3KAc is abundant at the promoters of active genes differentially expressed at higher levels in adherent strains.

In most eukaryotes, acetylation of histones located in gene promoters precedes the activation of many genes by creating a suitable chromatin environment that allows for the assembly of the transcriptional machinery. As this epigenetic mark has yet to be described in *T. vaginalis*, we first carried out an immunofluorescence assay using antibodies against H3KAc to investigate whether histone 3 lysine acetylation is present in the parasite's nucleus. The commercial antibody used specifically recognizes histone H3 acetylated at lysines 9, 14, 18, 23 or 27 (Abcam). Importantly, the acetylated lysine residues in *T. vaginalis* core H3 protein sequences are 100% conserved comparing to the human peptide used to generate the antibody (Fig. 1S). As expected for histones labeling, we found that the signal was restricted to the

nucleus of two strains (G3 and B7268) tested, with no cross-reaction with cytosolic proteins (Fig. 1A).

To elucidate whether the status of H3KAc is correlated with active transcription of genes, we developed a chromatin immunoprecipitation (ChIP) assay for *T. vaginalis.* The abundance of histone 3 lysine acetylation was monitored along BAP1 and BAP2 genes (de Miguel *et al.*, 2010; Riestra *et al.*, 2015) in high-adherent (B7268) versus less-adherent (G3) strains (Fig. 1C). These genes were selected as they present a marked difference in expression between the analyzed strains when analyzed by qPCR (Fig. 1B); with both being abundantly expressed in the B7268 and poorly expressed in the G3 strain. We designed sets of primers targeting the promoter (-0.5 kbp from the TSS), the proximities of the 5'UTR (+0.5 kbp from the TSS) and the coding sequence (+2.0 kbp from the TSS) for each BAP gene (Fig. 1C). Interestingly, we found a strict positive correlation between the level of BAP expression (Fig. 1B) and the abundance of H3KAc in the analyzed strain (Fig. 1C). High expression of the BAP genes is accompanied by high occupancy of H3KAc in the proximities of their 5'UTRs in highly-adherent B7268 strain. Conversely, low expression of BAPs genes correlates with low abundancy of H3KAc in less-adherent G3 strain (Fig. 1B-C). Notably, the greatest variation on the abundance of H3KAc was observed in the proximity of the 5'UTRs; where the Inr regulatory motifs are located for both BAPs. In order to exclude the possibility that variations on transcription between strains could be consequence of nucleotide differences on the promoter region of BAP1 and BAP2 genes, we have amplified and sequenced ~300 bp of their promoter regions in B7268 and compared to G3 strain (genome sequence). No differences were observed at nucleotide level among strains within BAP2 promoter and only two nucleotides differed in the promoter region of BAP1 (Fig. 2S). Importantly, those two nucleotides are not located within the Inr sequence.

**Histone acetylation regulates gene transcription**

Histone acetylation is strictly controlled by the concerted activity of histone acetylases (HATs) and deacetylases (HDACs), which has been extensively demonstrated to act as central organizers of chromatin remodeling and gene transcription. Based on the correlation of higher BAP expression with higher H3KAc abundance (Fig. 1), we predicted that a correct balance between acetylation and deacetylation may play a critical role in *T. vaginalis* expression and pathogenesis. Hence, we evaluated whether increased acetylation levels regulate gene transcription by using a class I and II HDAC inhibitor, trichostatin A (TSA). Firstly, we demonstrated by immunofluorescence assays, using an anti-H3KAc antibody, that increasing doses of TSA leads into an increased abundance of histone acetylation at the nucleus (Fig. 2A-B). In agreement with this, western blot assay demonstrated that the treatment of G3 strain with 400 nM of TSA lead to an increased histone acetylation (Fig. 2C). Importantly, no-change was observed on the abundance of the acetylated tubulin, demonstrating the specificity of the TSA treatment (Fig. 2C). Additionally, it should be noted that incubation of parasites (14 h) with up to 800 nM TSA did not affect parasite viability (Fig. 3S). We then analyzed whether TSA treatments modified the levels of mRNA expression of five genes (BAP1, BAP2, TVAG_110300, TvTSP2 and TvTSP8) enriched in the surface proteome of highly adherent compared to weakly-adherent strains (de Miguel *et al.*, 2010). RNA samples collected from G3 treated with different concentration of TSA were analyzed by RT-qPCR and compared with the expression of the genes in untreated G3 and B7268 strains (Fig. 3). The expression of all five genes was strikingly increased (in a dose-response manner with the exception of TVAG_110300) after TSA treatment. Concentration of TSA as low as 100 nM produced a substantial increase in the expression of all the analyzed genes (Fig. 3). Interestingly, BAP2 and TVAG_110300 were the most up-regulated, increasing ~250 and 330-fold respectively when parasites were treated with 400 nM TSA.

(Fig. 3). It is notable that the expression of some genes in G3 TSA-treated parasites resembles what is observed for the highly adherent B7268 strain. We then evaluate the effect of TSA in the adherent strain B7268 which already have high level of histone acetylation. Interestingly, only a ~2.3 and 1.9-fold increase in BAP1 and BAP2 transcription upon treatment with 400 nM TSA when compared to untreated B7268 parasites is observed (Fig. 4S). As control, the expression of a constitutively expressed hydrogenosomal gene (Hmp24) was analyzed (Fig. 3). Importantly, only a minimal change in Hmp24 expression was observed upon TSA treatment; highlighting the specificity of the treatment. These results demonstrate for the first time, that histone acetylation/deacetylation play an important role regulating gene transcription in *T. vaginalis*.

In concordance, ChIP analysis indicate that TSA treatment of G3 strain results in an increase in the abundance of H3KAc at the BAP1 and BAP2 loci which are normally hypoacetylated in this strain (Fig. 4). Moreover, the abundance of H3KAc in some regions of BAP1 and BAP2 genes on G3 TSA-treated parasites is comparable, or in some cases even higher, to the level of H3KAc observed in the highly adherent B7268 strain. Collectively, our data provide strong evidence that treatment of a weakly adherent strain with TSA induced histone hyperacetylation and up-regulation of genes encoding proteins normally enriched on the surface of highly adherent strains (de Miguel *et al.*, 2010).

**TSA treatment relaxes chromatin structure**

It has been well demonstrated in different biological systems that histone acetylation plays a crucial role in relaxing chromatin structure, thus allowing DNA-bound transcription factors to exert higher transcriptional potentials (Asahara *et al.*, 2001; Dilworth *et al.*, 2004). In order to isolate nucleosome-depleted DNA from *T. vaginalis* chromatin, formaldehyde assisted

isolation of regulatory elements (FAIRE) was perfomed, as chromatin isolated using FAIRE is negatively correlated with nucleosome occupancy (Giresi *et al.*, 2007). Chromatin of TSA treated and untreated control G3 parasites was crosslinked with formaldehyde *in vivo*, sheared by sonication, phenol-chloroform extracted and analyzed by qPCR. Interestingly, the DNA recovered in the aqueous phase after TSA treatment is 4- and 3-fold enriched for BAP1 and BAP2 genes, respectively, compared with untreated cells (Fig. 5). Hence, our results reflect that TSA treatment increase histone acetylation leading to an open conformation of the chromatin structure.

**Increased histone acetylation enables binding of IBP39 transcription factor**

Bioinformatic analyses indicates that the DNA-binding transcription factor IBP39 activates transcription on ~75% of known *T. vaginalis* genes (Smith and Johnson, 2011; Smith *et al.*, 2011) and is thus a key factor regulating gene expression. However, the relationship between the accessibility of IBP39 to the Inr regulatory element to which it binds (Liston *et al.*, 2001) and the chromatin structure has not been elucidated. To this end, we evaluated the relationship between histone acetylation, affected by the TSA treatment, and the binding of IBP39 to target genes. ChIP assays were performed using an anti-HA antibody in parasites transfected with IBP39-HA or empty vector (EpNeo) upon treatment with TSA. As expected, IBP39-HA is immunodetected at the nucleus of transfected parasites, and no signal is observed in parasites transfected with EpNeo (Fig. 6A). BAP1 and BAP2 genes were selected as potential IBP39-targets as bioinformatic analysis demonstrated that both genes contain a conserved Inr motif (TCA+1Py(T/A)) in the proximities of their TSS (Fig. 6B). Our results revealed that IBP39-HA binding on the proximities of BAP1 and BAP2 TSS region increased drastically after treatment with TSA (Fig. 6C), unlike that observed for control parasites

transfected with the EpNeo vector. As a control, we evaluated nonspecific binding in +2 Kbp regions of BAP1 and BAP2 genes as these regions lack the Inr regulatory element (Fig. 6C). The results indicated that the accessibility of IBP39 to the Inr binding sites is strictly dependent of the acetylated state of the histones and the chromatin structure; suggesting that H3KAc is a permissive histone modification associated with transcriptional activation.

**Histone acetylation regulates parasite aggregation and adherence to cervical cells**

We have previously noted that highly-adherent strains generally aggregate in cell culture, in contrast to less-adherent strains (such as G3) (Coceres *et al.*, 2015). Moreover, this observation has been correlated with the ability of the strain to adhere and be cytotoxic to host cells (Lustig *et al.*, 2013; Coceres *et al.*, 2015). Based on our observation that treatment with TSA led into an upregulation of genes that encode for surface proteins in G3 strain that are over expressed in highly adherent strains (Fig. 3), we tested whether this effect is accompanied with an increase in the aggregation capacity of those parasites. Interestingly, we observed that in the presence of TSA the G3 strain exhibited a dose-dependent increase in clump formation (Fig. 7A-B).

Although aggregation (parasite to parasite) and cytoadherence (parasite to host) are two different mechanisms that might be differentially regulated, the more the parasite aggregate to one another, the more adherence, as one adhering parasite carries others to the surface of the host cells. Hence, to determine if clump formation is accompanied with an increase capability of the parasites to adhere to the host cells, we performed adherence assays with host cells (Fig. 7C). Our results demonstrated that 400 nM TSA treatment resulted in an increase of attachment to host cells ~2.5-fold, compared to non-treated G3 parasites (Fig.

7C). These results unambiguously demonstrated for first time that epigenetic changes may be a key factor in regulating the adherence capacity of different *T. vaginalis* strains.

## DISCUSSION

Although epigenetic control of virulence factors has been well demonstrated in several microbial pathogens (Freitas-Junior *et al.*, 2005; Saksouk *et al.*, 2005; Janzen *et al.*, 2006; Sullivan *et al.*, 2006; Ehrenkaufer *et al.*, 2007; Iyer *et al.*, 2008; Tonkin *et al.*, 2009; Chaal *et al.*, 2010; Sonda *et al.*, 2010; Coleman *et al.*, 2014); nothing is known about the epigenetic mechanisms required to regulate transcription of genes in *T. vaginalis*. Histones are crucial players in epigenetic regulation and a basal repertoire of histones may thus be inherent to all eukaryotes (Postberg *et al.*, 2010). The *T. vaginalis* genome (http://TrichDB.org) contains a large number of histone genes (Table 2S), with most organized as gene pairs in a head-to-head manner (Cong *et al.*, 2010). Histones generally carry post-translational modifications, primarily either acetylations or methylations. Here, we analyzed the role of histone acetylation in *T. vaginalis* and found that it plays a key role regulating transcription and parasite pathogenesis. The importance of histone acetylation in the transcriptional regulation lies in its reversible property depending on various histone acetylases (HATs) and histone deacetylases (HDACs). Analysis of *T. vaginalis* genome reveals a family of at least 20 putative HATs and 21 HDACs, which can be grouped into different classes based upon similarity to human homologues (Table S2). The expansion of genes encoding both HATs and HDACs (Iyer *et al.*, 2008; Religa and Waters, 2012) might be signifying the importance of epigenetic regulation in *T. vaginalis.*

Trichostatin A treatment might be modifying the abundance of histone acetylation in numerous genes involved in different processes and some of them could be related to

regulation of parasite pathogenesis. Here, we have specifically demonstrated that the level of H3 acetylation of BAP1 and BAP2 genes correlates with their level of expression in strains with different adherence capacities. This is in agreement with the role of these proteins related with the adherence of this extracellular parasite to host cells (de Miguel *et al.*, 2010). High expression of the BAPs genes is accompanied by high occupancy of H3KAc in highly-adherent B7268 strain and conversely, low expression of BAPs genes correlates with low levels of H3KAc in less-adherent G3 strain. Additionally, hyper acetylation induced by HDAC inhibitor TSA increased expression of poorly expressed BAP1 and BAP2 genes in the less-adherent G3 strain. These results indicate that these genes, involved in parasite adherence, are at least partially regulated by acetylation/deacetylation of chromatin. In other protozoan parasites histone modifications has been shown to play important roles in life cycle progression, gene expression and antigenic variation (Freitas-Junior *et al.*, 2005; Janzen *et al.*, 2006; Ehrenkaufer *et al.*, 2007; Tonkin *et al.*, 2009; Chaal *et al.*, 2010). Similar to our data, treatment with drugs that disturb histone acetylation affected overall gene expression and stage-conversion in *Entamoeba histolytica* (Ehrenkaufer *et al.*, 2007) and *Toxoplasma* (Saksouk *et al.*, 2005), respectively. Additionally, differential acetylation in the promoters of stage-specific genes during stage conversion has been demonstrated in *Toxoplasma* (Saksouk *et al.*, 2005). Importantly, in *Plasmodium falciparum*, the *var* gene family, involved in antigenic variation, is transcribed in a mutually exclusive fashion and the NAD+-dependent histone deacetylase family cooperates to achieve silencing of the full *var* gene repertoire (Tonkin *et al.*, 2009). These observations highlight that epigenetic factors are key determinants of pathogenicity in malaria infection (Tonkin *et al.*, 2009). In concordance with that previously observed in other eukaryotic pathogens, we found that gene expression of surface genes appears to be sensitive to the acetylation status of core histones in *T. vaginalis*.

To date, several aspects of *T. vaginalis* transcriptional regulation have been explored. In this sense, most of the studies regarding gene expression in *T. vaginalis* have been centered on the finding of transcription factors acting at the promoter region of protein-coding genes (Quon *et al.*, 1994; Liston and Johnson, 1999; Smith and Johnson, 2011; Smith *et al.*, 2011), however the relationship between basal transcription machinery and chromatin structure has not been previously analyzed. A metazoan like TATA promoter element appears to be absent in trichomonad promoters (Smith and Johnson, 2011) and an initiator (Inr) sequence has been identified as the only known core-promoter-element in this organism (Liston and Johnson, 1999). The Inr promoter element was found in ~75% of the protein coding genes and the majority of these utilize the Inr to direct transcription initiation; supporting its central role in gene expression (Schumacher *et al.*, 2003). An Inr-binding protein, IBP39, was isolated and its fundamental role in transcription has been demonstrated (Liston *et al.*, 2001).

The level of acetylation of lysine residues on histone tails has been demonstrated to dictate chromatin structure (Boeger *et al.*, 2003; Reinke and Hörz, 2003; Shahbazian and Grunstein, 2007) and provide landmarks necessary for gene transcription or silencing (Strahl and Allis, 2000; Millar and Grunstein, 2006). Here, we show that IBP39 binding to the Inr element is strictly dependent of the acetylated state of the histones and the chromatin structure. Specifically, increased histone acetylation opens the chromatin structure and favor the binding of this transcription factor to the Inr motif at BAP1 and BAP2 genes allowing gene transcription. In contrast, low acetylation of histones results in a closed chromatin structure that blocks the IBP39 binding to the Inr motif. Thus, this is the first study that exemplifies the mutual dependence and complex crosstalk among epigenetic players, chromatin structure and basal gene expression machinery on *T. vaginalis*. Interestingly, parasitic protists, irrespective of their phylogeny, typically possess fewer specific transcription factors (Iyer *et al.*, 2008).

This observation, in concordance with our data, suggest that transcriptional regulation in parasites may heavily rely on epigenetic regulation.

As an obligate extracellular parasite, the surface of *T. vaginalis* is likely to function in multiple ways to establish, maintain, and modulate infection (Ryan *et al.*, 2011). Interestingly, we demonstrated that histone acetylation can regulate the expression of genes encoding membrane or membrane associated proteins that are implicated in parasite aggregation and adhesion to host cells (de Miguel *et al.*, 2010; Coceres *et al.*, 2015). Specifically, we found that TSA treatment of a weakly adherent strain lead into the upregulation of genes encoding proteins differentially expressed on the surface of highly adherent strains (de Miguel *et al.*, 2010). Interestingly, our data also revealed that adherence and aggregation of *T. vaginalis* is highly dependent on the acetylated state of histones, as increased histone acetylation leads to an increase in parasite aggregation and adherence to host cell. These observations are consistent with increased expression of surface associated genes after TSA treatment being the responsible, at least partially, for increased parasite clumping and adhesion of the parasite to human host cells. Moreover, the increased TSP8 expression and increased parasite aggregation in TSA treated-parasites (Coceres *et al.*, 2015) is in agreement with our recent observation that transfection of parasites with surface-localized TSP8 promotes the ability of a non-clumping parasite strain to aggregate, (Coceres *et al.*, 2015). Taking together, these data support that an increase in TSP8, BAP1 and BAP2 expression, observed upon TSA treatment may be facilitating, critical properties that play a role in parasite pathogenesis.

This work identifies and highlight for the first time the importance of the epigenetic contribution in the control of gene expression and *T. vaginalis* pathogenesis. A better understanding of the epigenetic differences between strains may account for the variation in

symptoms and disease progression observed in women infected by this pathogen and will provide insights into pathogenesis and possibly new avenues for diagnosis and therapy.

## MATERIALS AND METHODS

### *Parasites, cell cultures and media*

*Trichomonas vaginalis* strains B7268 (Upcroft and Upcroft, 2001) and G3 (ATCC PRA-98, Kent, UK) were cultured in TYM medium supplemented with 10% horse serum, penicillin and streptomycin (Invitrogen) (Clark and Diamond, 2002). Parasites were grown at 37°C and passaged daily. The human HeLa cells (ATCC® CCL2™) were grown in DMEM complemented with 10% bovine fetal serum, penicillin and streptomycin (Invitrogen) and cultured at 37°C/5% $CO_2$.

### *Immunolocalization experiments*

Parasites in the absence of host cells were incubated at 37°C on glass coverslips as previously described (de Miguel *et al.*, 2010) for 4 hours. The parasites were then fixed and permeabilized in cold methanol for 10 min. The cells were washed and blocked with 5% FBS in PBS for 30 min, incubated with a 1:500 dilution of anti-H3KAc (Anti-Histone H3 acetyl K9 + K14 + K18 + K23 + K27 antibody - ChIP Grade, ab47915, Abcam, USA) or anti-HA (Covance, USA) primary antibody diluted in PBS plus 2% FBS, washed and then incubated with a 1:5000 dilution of Alexa Fluor conjugated secondary antibody (Molecular Probes). The coverslips were mounted onto microscope slides using ProLong Gold antifade reagent with 4′, 6′-diamidino-2-phenylindole (Invitrogen). All observations were performed on a

Nikon E600 epifluorescence microscope. Adobe Photoshop (Adobe Systems) was used for image processing.

***Chromatin immunoprecipitation (ChIP)***

*T. vaginalis* was grown overnight to a density of ~1 x $10^6$ cells/ml and separated in aliquots of 2.5x$10^8$ parasites. Cells were centrifuged and resuspended in nuclear extraction buffer (0.5% NP-40; 0.25% TX-100; 10 mM Tris-HCl; 3 mM $CaCl_2$; 250 mM Sucrose; 1mM DTT; 200 mM PMSF; EDTA-free Protease Inhibitors (PI)) plus 1% formaldehyde to crosslink proteins and DNA. Cross-linking was stopped by the addition of 125 mM glycine, followed by incubation on nutator for 5 min at room temperature. Cells were harvested, washed in 1ml PBS/PI and resuspended in 1ml nuclear extraction buffer followed by homogenization in a dounce tube to help release the nuclei into the solution. After the centrifugation, the pellet was resuspended in SDS-lysis buffer (1% SDS; 50 mM Tris-HCl pH8.0; 10 mM EDTA; 1X PI) and 2 vol of ChIP dilution buffer (0.01% SDS; 1.2 mM EDTA; 16.7 mM Tris-HCl pH 8.0; 167 mM NaCl; 1 mM DTT; 0.2 mM PMSF; 1X PI) was added. Chromatin was sheared to 300–800-bp fragments using a water-bath sonicator (Amp: 30%, Cycle: 30 sec ON and 30 sec OFF, Time: 15 min) and centrifuged, and the supernatant was split in three tubes for input sample, mock control (rabbit anti-IgG, Abcam), and target antibodies mouse anti-HA (Covance, USA) or rabbit anti-H3KAc (Abcam) all bound to protein A magnetic beads (Invitrogen). IgG and target antibodies (5 µg) were incubated nutating overnight at 4°C and after extensive washes with RIPA buffer (50 mM Hepes-KOH pH 8.0; 500 mM LiCl; 1 mM EDTA; 1% NP-40; 0.7% Na-deoxycholate; 1 mM DTT; 0.2 mM PMSF; 1X PI), the complexes were resuspended in elution buffer (50 mM Tris-HCl pH 8.0; 10 mM EDTA; 1% SDS). The magnetic beads were eluted by incubating at 65°C for 15 min, vortexing every 2

min, and then spun down at 16,000 x g for 1 min at room temperature. The supernatant was reverse cross-linked by heating at 65°C overnight. Immunoprecipitated DNA was treated with 0.2 µg/ml RNase A and 0.2 µg/ml proteinase K both for 1 hr. Phenol/chloroform/isoamyl alcohol extraction was performed followed by EtOH precipitation. Finally, the purified DNA was used as a template for qPCR analyzes. Binding to three different regions along the BAP1 (TVAG_166850) and BAP2 (TVAG_244130) genes were analyzed: -0.5kb from TSS; +0.5kb from TSS; and +2.0kb from TSS. The primers used are described in S1 Table. Each sample was loaded in triplicate and the results were expressed as % input relative to -0.5kb from TSS region of G3 strain.

***Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE)***

*T. vaginalis* was treated with 400nM TSA for 14 h in triplicate and grown to a density of 1 x $10^6$ cells/ml. Cells were crosslinked, lysated and sonicated as described in ChIP assays. Sonicated DNA was split in two tubes: input and FAIRE DNA sample. Input was treated with RNAse-I and proteinase K and then incubated overnight at 65°C to reverse crosslinking. FAIRE DNA was subjected to DNA extraction with an equal amount of phenol/chloroform/isoamyl. DNA was then ethanol precipitated, treated 30 min at 37°C with RNAse-I and 1 h at 55°C with proteinase K. After purification, FAIRE DNA was incubated overnight at 65°C to reverse any DNA-DNA crosslinks. The input DNA was then purified using phenol/chloroform/isoamyl extraction followed by ethanol precipitation. Finally, the level of open chromatin were examined by qPCR. Inr promoter regions of BAP1 (TVAG_166850) and BAP2 (TVAG_244130) genes were analyzed. The primers used are described in S1 Table. Each replicate sample was loaded in triplicate and the results were expressed as % input relative to untreated G3 strain.

***Quantitative PCR (qPCR)***

Total RNA was extracted from ~4 x $10^6$ *T. vaginalis* using TRIzol (Invitrogen) following the manufacturer's instructions. Total RNA was treated with amplification grade DNase I (Invitrogen) and reverse transcribed using SuperScript II reverse transcriptase and oligo (dT) primers (Invitrogen). Real-time PCRs were performed using Brilliant SYBR Green qPCR Master Mix (Roche), a 150-450 nM concentration of each primer, and 200-500 ng of cDNA in a 20 µl reaction volume using a Stratagene Mx3005P$^{TM}$ system (Agilent Technologies). Parallel reactions performed without reverse transcriptase were included as negative controls. Using data from the exponential phase of the qPCR, threshold cycle (CT) base lines were set according to manufacturer protocols. Data from different samples were interpolated from standard curves established for each primer set and then normalized against the housekeeping alpha tubulin gene (dos Santos *et al.*, 2015). The expression of housekeeping gene does not seem to change upon TSA treatment (data not shown). Every experimental and standard curve sample was tested in triplicates in three independent experiments. The primers used were listed in S1 Table.

***IBP39 expression in T. vaginalis***

The IBP39 gene was cloned into the Master-Neo-$(HA)_2$ plasmid (Delgadillo *et al.*, 1997). Electroporation of *T. vaginalis* strain G3 was carried out as described previously (Delgadillo *et al.*, 1997) with 50 µg of circular plasmid DNA. Transfectants were selected with 100 µg/ml G418 (Sigma).

***Parasite aggregation***

Parasite aggregation was analyzed in anaerobic conditions when parasites reach a concentration of $10^6$ parasites/ml using a Nikon E600 epifluorescence microscope with a magnification of 10X. Adobe Photoshop (Adobe Systems) was used for image processing. Quantification of clumps in thirty fields were analyzed per treatment.

***Adherence assay***

A modified version of an *in vitro* assay to qualify the binding of *T. vaginalis* to host cell monolayers (Bastida-Corcuera *et al.,* 2005) was performed. Briefly, HeLa cells were seeded on 12-mm coverslips in 24-well plates at 3 x $10^5$ cells/well in culture medium and grown to confluence at 37 °C in 5% $CO_2$ for 2 days. Cell monolayers were washed before the addition of parasites. *T. vaginalis* was labeled with 10 mM CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) (Invitrogen), and $10^5$ labeled parasites in 0.5 ml of keratinocyte medium (Invitrogen) were added (1:3 parasite:host cell ratio) in triplicate. Plates were incubated at 37°C in 5% $CO_2$ for 30 min. Coverslips were subsequently washed in PBS, fixed with 4% paraformaldehyde, and mounted on slides with Mowiol (Calbiochem). Twenty 10X magnification fields were analyzed per coverslip with three coverslips per treatment.

***Statistical analyses***

Graphs were made and statistical analyses performed using GraphPad Prism. Independent experiments were performed a minimum of 3 times with at least three technical replicates per experiment. Two sample t-tests or ANOVA were used to determine significance. Data are expressed as standard error of the mean (± SEM).

**ACKNOWLEDGEMENTS**

We thank our colleagues in the lab for helpful discussions. This research was supported with a grant from the International Society for Infectious Diseases (NdM) and ANPCyT grant BID PICT 2015-2118 (NdM). NdM and PSM are researchers from the National Council of Research (CONICET) and UNSAM. YRN and AL are a PhD fellow from CONICET. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**REFERENCES**


Asahara, H., Santoso, B., Guzman, E., Du, K., Cole, P.A., Davidson, I., and Montminy, M. (2001) Chromatin-dependent cooperativity between constitutive and inducible activation domains in CREB. *Mol Cell Biol* **21**: 7892–7900.

Bastida-Corcuera, F.D., Okumura, C.Y., Colocoussi, A., and Johnson, P.J. (2005) Trichomonas vaginalis lipophosphoglycan mutants have reduced adherence and cytotoxicity to human ectocervical cells. *Eukaryot Cell* **4**: 1951–1958.

Boeger, H., Griesenbeck, J., Strattan, J.S., and Kornberg, R.D. (2003) Nucleosomes unfold completely at a transcriptionally active promoter. *Mol Cell* **11**: 1587–1598.

Carlton, J.M., Hirt, R.P., Silva, J.C., Delcher, A.L., Schatz, M., Zhao, Q., *et al.* (2007) Draft genome sequence of the sexually transmitted pathogen Trichomonas vaginalis. *Science* **315**: 207–212.

Chaal, B.K., Gupta, A.P., Wastuwidyaningtyas, B.D., Luah, Y.H., and Bozdech, Z. (2010) Histone deacetylases play a major role in the transcriptional regulation of the Plasmodium falciparum life cycle. *PLoS Pathog* **6**: e1000737.

Clark, C.G., and Diamond, L.S. (2002) Methods for cultivation of luminal parasitic protists of clinical importance. *Clin Microbiol Rev* **15**: 329–41.

Coceres, V.M., Alonso, A.M., Nievas, Y.R., Midlej, V., Frontera, L., Benchimol, M., *et al.* (2015) The C-terminal tail of tetraspanin proteins regulates their intracellular distribution in the parasite Trichomonas vaginalis. **17**: 1217–1229.

Coleman, B.I., Skillman, K.M., Jiang, R.H.Y., Childs, L.M., Altenhofen, L.M., Ganter, M., *et al.* (2014) A Plasmodium falciparum Histone Deacetylase Regulates Antigenic Variation and Gametocyte Conversion. *Cell Host Microbe* .

Cong, P., Luo, Y., Bao, W., and Hu, S. (2010) Genomic organization and promoter analysis of the *Trichomonas vaginalis* core histone gene families. *Parasitol Int* **59**: 29–34.

Delgadillo, M.G., Liston, D.R., Niazi, K., and Johnson, P.J. (1997) Transient and selectable transformation of the parasitic protist Trichomonas vaginalis. *Proc Natl Acad Sci U S A* **94**: 4716–4720.

Dilworth, F.J., Seaver, K.J., Fishburn, A.L., Htet, S.L., and Tapscott, S.J. (2004) In vitro transcription system delineates the distinct roles of the coactivators pCAF and p300 during MyoD/E47-dependent transactivation. *Proc Natl Acad Sci U S A* **101**: 11593–11598.

Duraisingh, M.T., Voss, T.S., Marty, A.J., Duffy, M.F., Good, R.T., Thompson, J.K., *et al.* (2005) Heterochromatin Silencing and Locus Repositioning Linked to Regulation of Virulence Genes in Plasmodium falciparum. *Cell* **121**: 13–24.

Ehrenkaufer, G.M., Eichinger, D.J., and Singh, U. (2007) Trichostatin A effects on gene expression in the protozoan parasite Entamoeba histolytica. *BMC Genomics* **8**: 216.

Fichorova, R.N. (2009) Impact of T. vaginalis infection on innate immune responses and reproductive outcome. *J Reprod Immunol* **83**: 185–189.

Freitas-Junior, L.H., Hernandez-Rivas, R., Ralph, S.A., Montiel-Condado, D., Ruvalcaba-Salazar, O.K., Rojas-Meza, A.P., *et al.* (2005) Telomeric Heterochromatin Propagation and Histone Acetylation Control Mutually Exclusive Expression of Antigenic Variation Genes in Malaria Parasites. *Cell* **121**: 25–36.

Gander, S., Scholten, V., Osswald, I., Sutton, M., and Wylick, R. van (2009) Cervical Dysplasia and Associated Risk Factors in a Juvenile Detainee Population. *J Pediatr Adolesc Gynecol* **22**: 351–355.

Giresi, P.G., Kim, J., McDaniell, R.M., Iyer, V.R., and Lieb, J.D. (2007) FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res* **17**: 877–85.

Gould, S.B., Woehle, C., Kusdian, G., Landan, G., Tachezy, J., Zimorski, V., and Martin, W.F. (2013) Deep sequencing of Trichomonas vaginalis during the early infection of vaginal epithelial cells and amoeboid transition. *Int J Parasitol* **43**: 707–719.

Horvathova, L., Safarikova, L., Basler, M., Hrdy, I., Campo, N.B., Shin, J.W., *et al.* (2012) Transcriptomic Identification of Iron-Regulated and Iron-Independent Gene Copies within the Heavily Duplicated Trichomonas vaginalis Genome. *Genome Biol Evol* **4**: 905–917.

Hsu, H.M., Ong, S.J., Lee, M.C., and Tai, J.H. (2009) Transcriptional Regulation of an Iron-Inducible Gene by Differential and Alternate Promoter Entries of Multiple Myb Proteins in the Protozoan Parasite Trichomonas vaginalis. *Eukaryot Cell* **8**: 362–372.

Huang, K.Y., Chen, Y.M., Fang, Y.K., Cheng, W.H., Cheng, C.C., Chen, Y.C., *et al.* (2014) Adaptive responses to glucose restriction enhance cell survival, antioxidant capability, and autophagy of the protozoan parasite Trichomonas vaginalis. *Biochim Biophys Acta - Gen Subj* **1840**: 53–64.

Huang, K.Y., Huang, P.J., Ku, F.M., Lin, R., Alderete, J.F., and Tang, P. (2012) Comparative transcriptomic and proteomic analyses of Trichomonas vaginalis following adherence to fibronectin. *Infect Immun* **80**: 3900–11.

Iyer, L.M., Anantharaman, V., Wolf, M.Y., and Aravind, L. (2008) Comparative genomics of transcription factors and chromatin proteins in parasitic protists and other eukaryotes. *Int J Parasitol* **38**: 1–31.

Janzen, C.J., Hake, S.B., Lowell, J.E., and Cross, G.A.M. (2006) Selective di- or trimethylation of histone H3 lysine 76 by two DOT1 homologs is important for cell cycle regulation in Trypanosoma brucei. *Mol Cell* **23**: 497–507.

Jenuwein, T., and Allis, C.D. (2001) Translating the histone code. *Science* **293**: 1074–1080.

Katan-Khaykovich, Y., and Struhl, K. (2002) Dynamics of global histone acetylation and deacetylation in vivo: Rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev* **16**: 743–752.

Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* **128**: 693–705.

Kusdian, G., and Gould, S.B. (2014) The biology of Trichomonas vaginalis in the light of urogenital tract infection. *Mol Biochem Parasitol* **198**: 92–99.

Leitsch, D., Kolarich, D., Binder, M., Stadlmann, J., Altmann, F., and Duchêne, M. (2009) Trichomonas vaginalis: Metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. *Mol Microbiol* **72**: 518–536.

Li, J., Lin, Q., Wang, W., Wade, P., and Wong, J. (2002) Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. 687–692.

Liston, D.R., Carrero, J.C., and Johnson, P.J. (1999) Upstream regulatory sequences required for expression of the Trichomonas vaginalis alpha-succinyl CoA synthetase gene. *Mol Biochem Parasitol* **104**: 323–9.

Liston, D.R., and Johnson, P.J. (1999) Analysis of a ubiquitous promoter element in a primitive eukaryote: early evolution of the initiator element. *Mol Cell Biol* **19**: 2380–8.

Liston, D.R., Lau, A.O., Ortiz, D., Smale, S.T., and Johnson, P.J. (2001) Initiator recognition in a primitive eukaryote: IBP39, an initiator-binding protein from Trichomonas vaginalis. *Mol Cell Biol* **21**: 7872–7882.

Lustig, G., Ryan, C.M., Secor, W.E., and Johnson, P.J. (2013) Trichomonas vaginalis contact-dependent cytolysis of epithelial cells. *Infect Immun* **81**: 1411–1419.

McClelland, R.S., Sangaré, L., Hassan, W.M., Lavreys, L., Mandaliya, K., Kiarie, J., *et al.* (2007) Infection with Trichomonas vaginalis Increases the Risk of HIV- 1 Acquisition. *J Infect Dis* **195**: 698–702.

Miguel, N. de, Lustig, G., Twu, O., Chattopadhyay, A., Wohlschlegel, J.A., and Johnson, P.J. (2010) Proteome analysis of the surface of Trichomonas vaginalis reveals novel proteins and strain-dependent differential expression. *Mol Cell Proteomics* **9**: 1554–1566.

Millar, C.B., and Grunstein, M. (2006) Genome-wide patterns of histone modifications in yeast. *Nat Rev Mol Cell Biol* **7**: 657–66.

Pol, B. Van Der, Kwok, C., Pierre- Louis, B., Rinaldi, A., Salata, R.A., Chen, P., *et al.* (2008) Trichomonas vaginalis Infection and Human Immunodeficiency Virus Acquisition in African Women. *J Infect Dis* **197**: 548–554.

Postberg, J., Forcob, S., Chang, W.J., and Lipps, H.J. (2010) The evolutionary history of histone H3 suggests a deep eukaryotic root of chromatin modifying mechanisms. *BMC Evol Biol* **10**: 259.

Quon, D. V, Delgadillo, M.G., Khachi, A., Smale, S.T., and Johnson, P.J. (1994) Similarity between a ubiquitous promoter element in an ancient eukaryote and mammalian initiator elements. *Proc Natl Acad Sci U S A* **91**: 4579–83.

Reinke, H., and Hörz, W. (2003) Histones Are First Hyperacetylated and Then Lose Contact with the Activated PHO5 Promoter. *Mol Cell* **11**: 1599–1607.

Religa, A.A., and Waters, A.P. (2012) Sirtuins of parasitic protozoa: In search of function(s). *Mol Biochem Parasitol* **185**: 71–88.

Riestra, A.M., Gandhi, S., Sweredoski, M.J., Moradian, A., Hess, S., Urban, S., and Johnson, P.J. (2015) A Trichomonas vaginalis Rhomboid Protease and Its Substrate Modulate Parasite Attachment and Cytolysis of Host Cells. *PLoS Pathog* **11**: e1005294.

Rojas, L., Sariego, I., Fraga, J., Sarría, C., Sarracent, J., and Sánchez, L. (2004) Use of in vitro cytoadherence assays in the comparative study of the virulence of isolates of Trichomonas vaginalis. *Parasitol Res* **93**: 332–337.

Ruijter, A.J.M. de, Gennip, A.H. van, Caron, H.N., Kemp, S., and Kuilenburg, A.B.P. van (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* **370**: 737–49.

Ryan, C.M., Miguel, N. de, and Johnson, P.J. (2011) Trichomonas vaginalis□: current understanding of host–parasite interactions. *Essays Biochem* **51**: 161–175.

Saksouk, N., Bhatti, M.M., Kieffer, S., Smith, A.T., Musset, K., Garin, J., *et al.* (2005) Histone-Modifying Complexes Regulate Gene Expression Pertinent to the Differentiation of the Protozoan Parasite Toxoplasma gondii Histone-Modifying Complexes Regulate Gene Expression Pertinent to the Differentiation of the Protozoan Parasite Toxoplasma go. *Mol Cell Biol* **25**: 10301–10314.

Santos, O. dos, Vargas Rigo, G. de, Frasson, A.P., Macedo, A.J., and Tasca, T. (2015) Optimal Reference Genes for Gene Expression Normalization in Trichomonas vaginalis. *PLoS One* **10**: e0138331.

Schones, D.E., Cui, K., Cuddapah, S., Roh, T.Y., Barski, A., Wang, Z., *et al.* (2008) Dynamic Regulation of Nucleosome Positioning in the Human Genome. *Cell* **132**: 887–898.

Schumacher, M.A., Lau, A.O.T., and Johnson, P.J. (2003) Structural Basis of Core Promoter Recognition in a Primitive Eukaryote. *Cell* **115**: 413–424.

Shahbazian, M.D., and Grunstein, M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* **76**: 75–100.

Smith, A., and Johnson, P. (2011) Gene expression in the unicellular eukaryote Trichomonas vaginalis. *Res Microbiol* **162**: 646–654.

Smith, A.J., Chudnovsky, L., Simoes-Barbosa, A., Delgadillo-Correa, M.G., Jonsson, Z.O., Wohlschlegel, J.A., and Johnson, P.J. (2011) Novel Core Promoter Elements and a Cognate Transcription Factor in the Divergent Unicellular Eukaryote Trichomonas vaginalis. *Mol Cell Biol* **31**: 1444–1458.

Sonda, S., Morf, L., Bottova, I., Baetschmann, H., Rehrauer, H., Caflisch, A., *et al.* (2010) Epigenetic mechanisms regulate stage differentiation in the minimized protozoan Giardia lamblia. *Mol Microbiol* **76**: 48–67.

Stark, J.R., Judson, G., Alderete, J.F., Mundodi, V., Kucknoor, A.S., Giovannucci, E.L., *et al.* (2009) Prospective study of Trichomonas vaginalis infection and prostate cancer incidence and mortality: Physicians' Health Study. *J Natl Cancer Inst* **101**: 1406–11.

Strahl, B.D., and Allis, C.D. (2000) The language of covalent histone modifications. *Nature* **403**: 41–45.

Sullivan, W.J., Naguleswaran, A., and Angel, S.O. (2006) Histones and histone modifications in protozoan parasites. *Cell Microbiol* **8**: 1850–61.

Sutcliffe, S., Alderete, J.F., Till, C., Goodman, P.J., Hsing, A.W., Zenilman, J.M., *et al.* (2009) Trichomonosis and subsequent risk of prostate cancer in the Prostate Cancer Prevention Trial. *Int J Cancer* **124**: 2082–2087.

Swygard, H., Miller, W.C., Kaydos-Daniels, S.C., Cohen, M.S., Leone, P.A., Hobbs, M.M., and Seña, A.C. (2004) Targeted Screening for Trichomonas vaginalis with Culture Using a Two-Step Method in Women Presenting for STD Evaluation. *Sex Transm Dis* **31**: 659–664.

Tonkin, C.J., Carret, C.K., Duraisingh, M.T., Voss, T.S., Ralph, S.A., Hommel, M., *et al.* (2009) Sir2 Paralogues Cooperate to Regulate Virulence Genes and Antigenic Variation in Plasmodium falciparum. *PLoS Biol* **7**: e1000084.

Turner, B.M. (2002) Cellular memory and the histone code. *Cell* **111**: 285–291.

Twu, O., Dessí, D., Vu, A., Mercer, F., Stevens, G.C., Miguel, N. de, *et al.* (2014) Trichomonas vaginalis homolog of macrophage migration inhibitory factor induces prostate cell growth, invasiveness, and inflammatory responses. *Proc Natl Acad Sci U S A* **111**: 1–6.

Upcroft, J.A., and Upcroft, P. (2001) Drug Susceptibility Testing of Anaerobic Protozoa. **45**: 1810–1814.

Yang, X.-J., and Seto, E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat Rev Mol Cell Biol* **9**: 206–218.

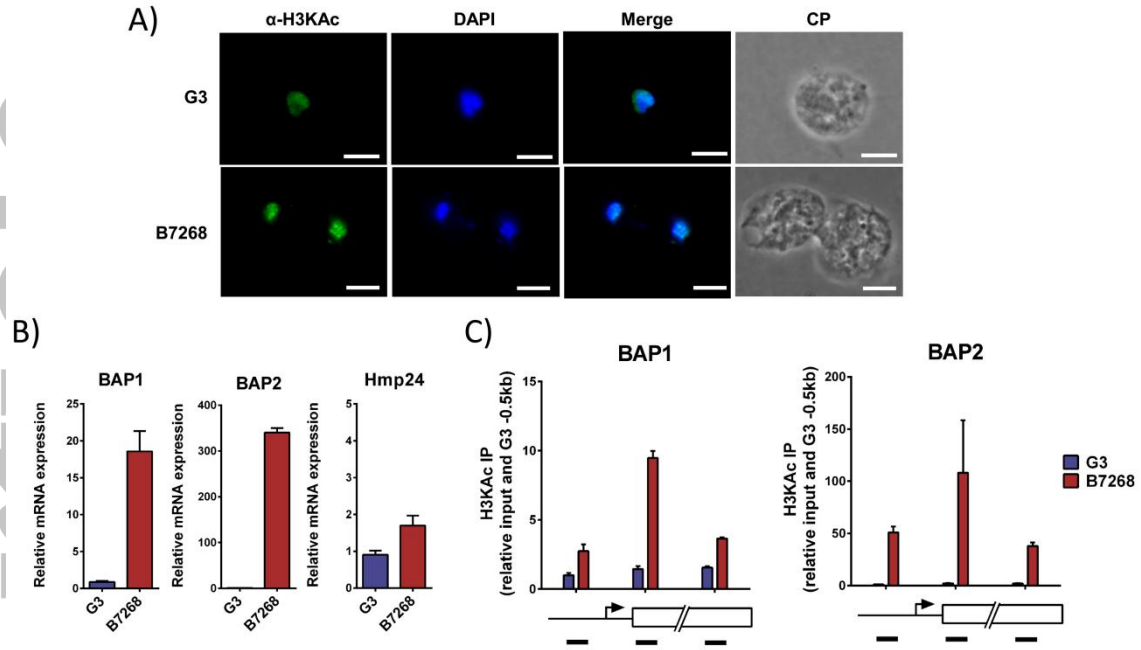


**Fig 1.** ***H3KAc is highly abundant at the promoters of BAPs genes in adherent strains.*** (A) Immunofluorescence assay showing the signal detected with anti-H3KAc antibody is restricted to the nuclei in G3 (less-adherent – upper panel) and B7268 (high-adherent –lower panel). Blue, DAPI nuclear staining; green, H3KAc; CP, Contrast Phase. (B) mRNA expression levels of BAP genes normalized against alpha-tubulin gene in G3 (blue) and B7268 (red) strains. As control, the expression of a constitutively expressed hydrogenosomal gene (Hmp24) was used, evidencing gene amplification in both of analyzed strains. Data are expressed as -fold increase compared with the G3 strain ± the standard deviation of the mean. (C) Abundance and distribution of histone acetylation within BAP1 (TVAG_166850) and BAP2 (TVAG_244130) genes in G3 (blue) and B7268 (red) analyzed by ChIP. Three regions along BAP1 and BAP2 genes (-0.5 kpb, +0.5 kbp and +2 kbp from TSS) were analyzed. Arrows depict the TSS, boxes represent the protein coding sequence and regions amplified are depicted as bold lines under each graph. Data are expressed as fold increase normalized to input and G3 -0.5 kbp.

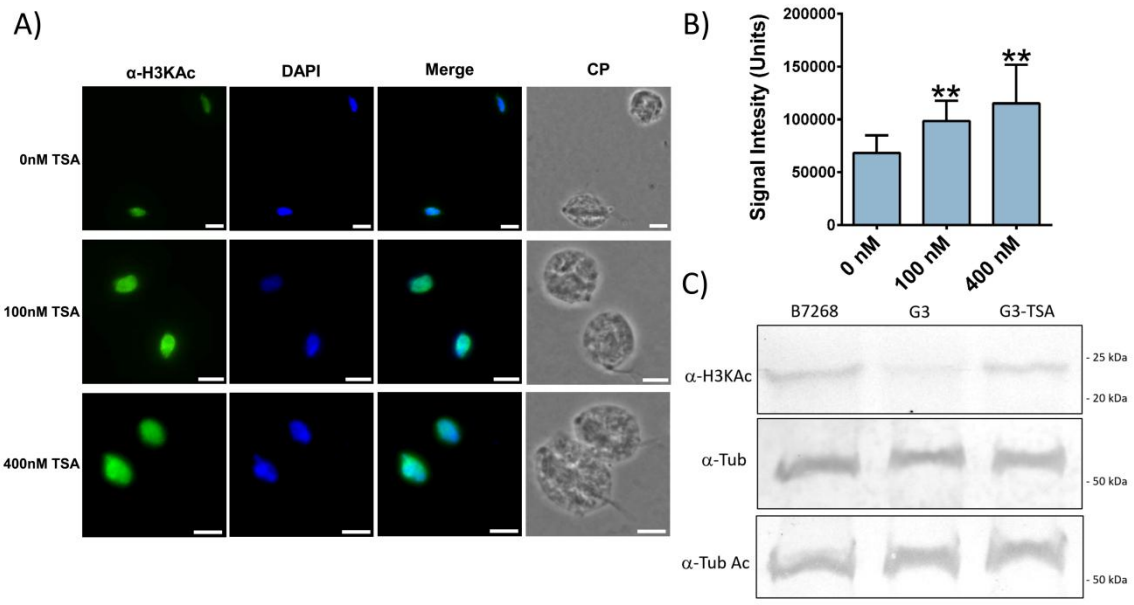


**Fig 2.** ***Histone hyperacetylation induced by HDAC inhibitor TSA.*** (A) Abundance of histone H3 acetylation was analyzed by immunofluorescence assays (IFAs) after treatment of G3 parasites with 100 nM or 400 nM of TSA. Parasites treated with the same volume of ethanol (TSA diluent) were used as control. Pictures were taken with the same exposure. The increased signal in TSA treated parasites indicated that H3 hyperacetylation is induced. Three independent experiments were performed with similar results. A representative experiment is shown. (B) Quantification of fluorescence intensity of IFAs. The signal in twenty parasites was analyzed in each case and quantified using ImageJ. ** $p < 0.001$. (C) Abundance of histone H3 acetylation was analyzed by western blot in B7268, G3 and after treatment of G3 parasites with 400 nM of TSA using an anti-acetylated Histone 3 antibody (α-H3KAc, upper panel). A western blot using anti-tubulin (α-tub, middle panel) was included to demonstrate that similar amount of protein was loaded in each lane. A western blot using an anti-acetylated tubulin (a-Tub Ac, lower panel) was included to demonstrate the specificity of the treatment.

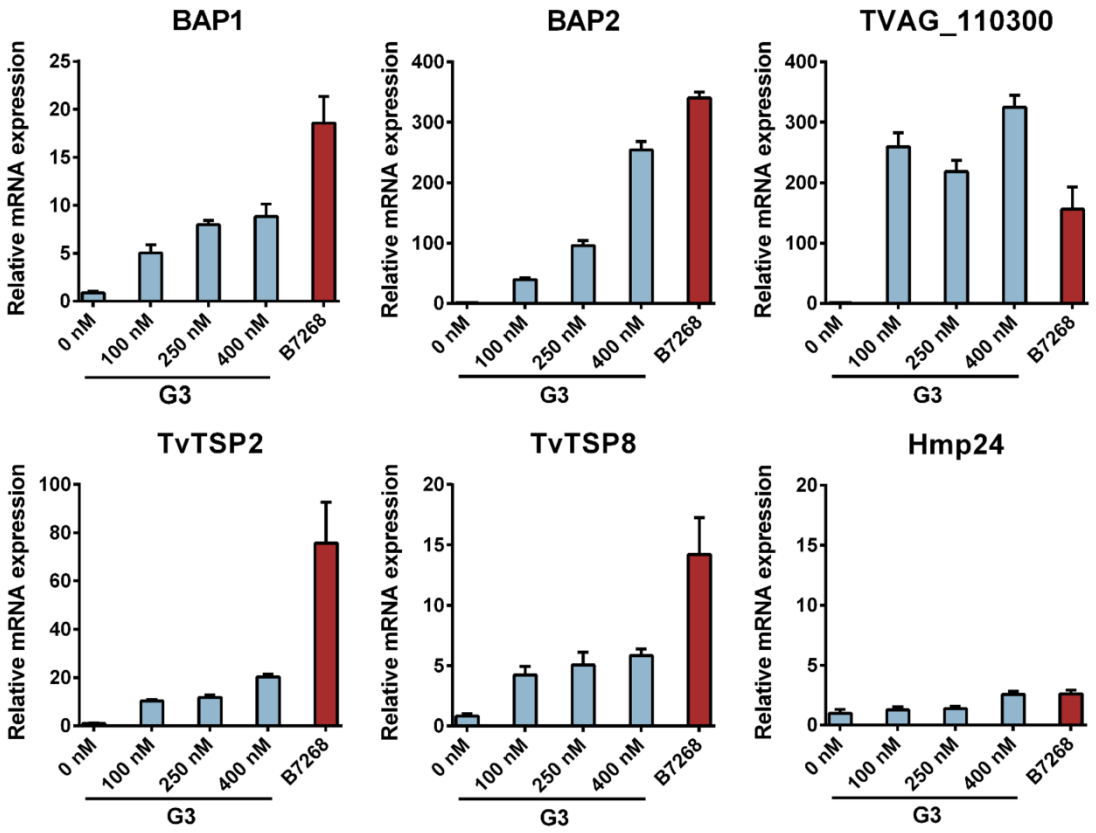


**Fig 3.** ***Expression analysis of five genes differentially expressed upon treatment with TSA.*** G3 parasites were exposed to increasing concentrations of TSA and expression was analyzed by RT-qPCR normalizing against tubulin housekeeping gene. Expression level of five transcripts previously identified as differentially expressed in G3 (less-adherent) and B7268 (high-adherent) were examined. As control, the Hmp24 gene was included. Data are expressed as fold increase compared to G3 0 nM ± the standard deviation of the mean. All tested mRNA levels are up-regulated after TSA treatment. All experiments were carried out in triplicate in three independent experiments. A representative experiment is shown.

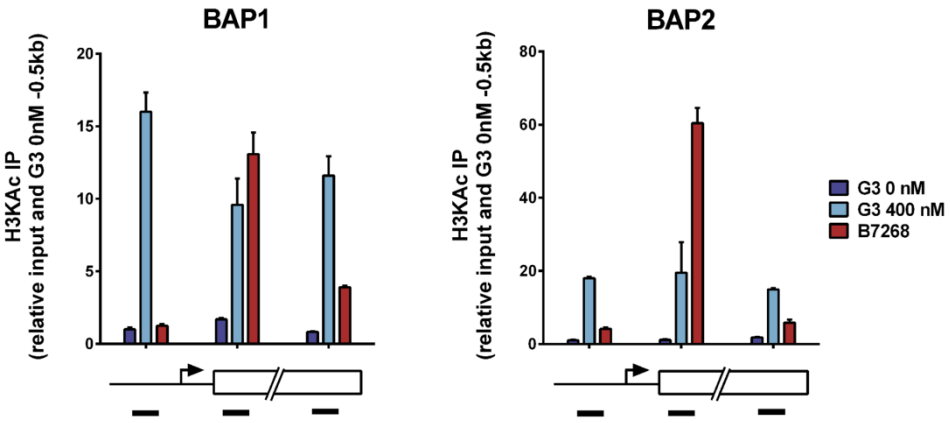


**Fig 4.** ***Abundance of histone H3 acetylation within BAP genes upon TSA treatment.*** Levels of histone H3 acetylation within BAP1 and BAP2 genes obtained by ChIP using an anti-H3KAc antibody. G3 untreated (blue), G3-TSA treated (light blue) and B7268 untreated (red) parasites were examined over the gene regions -0.5 kpb, +0.5 kbp and +2 kbp from TSS. H3KAc IP levels were normalized to input and G3 -0.5kb untreated control. Arrows depict the TSS, boxes represent the protein coding sequence and regions amplified are depicted as bold lines under each graph.

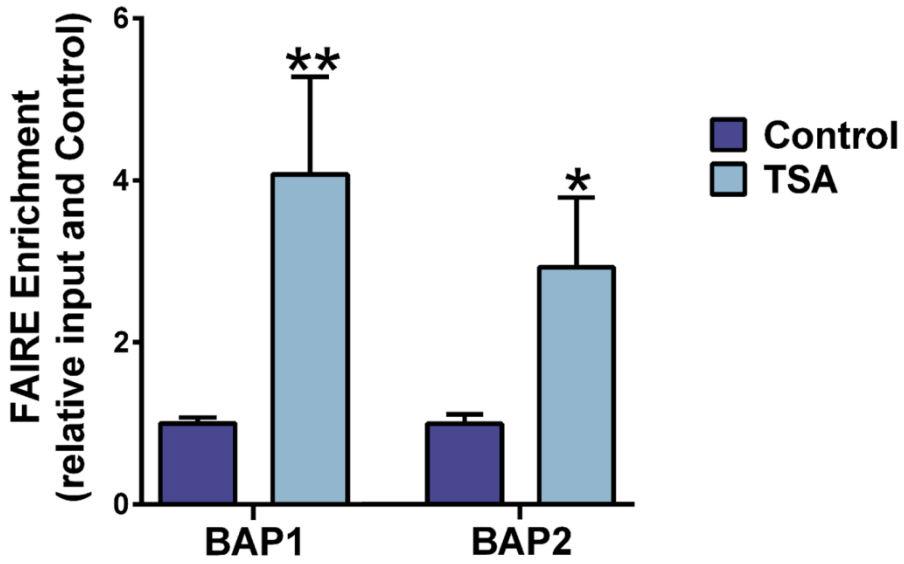


**Fig 5. TSA treatment affects distribution of nucleosomes.** G3 parasites treated with TSA (400nM) were subjected to FAIRE assay. The Y-axis indicates the enrichment of open chromatin relative to input and untreated samples. qPCR analysis used primers targeting the Inr region of BAP1 and BAP2 gene promoters. * $p < 0.05$, ** $p < 0.001$.

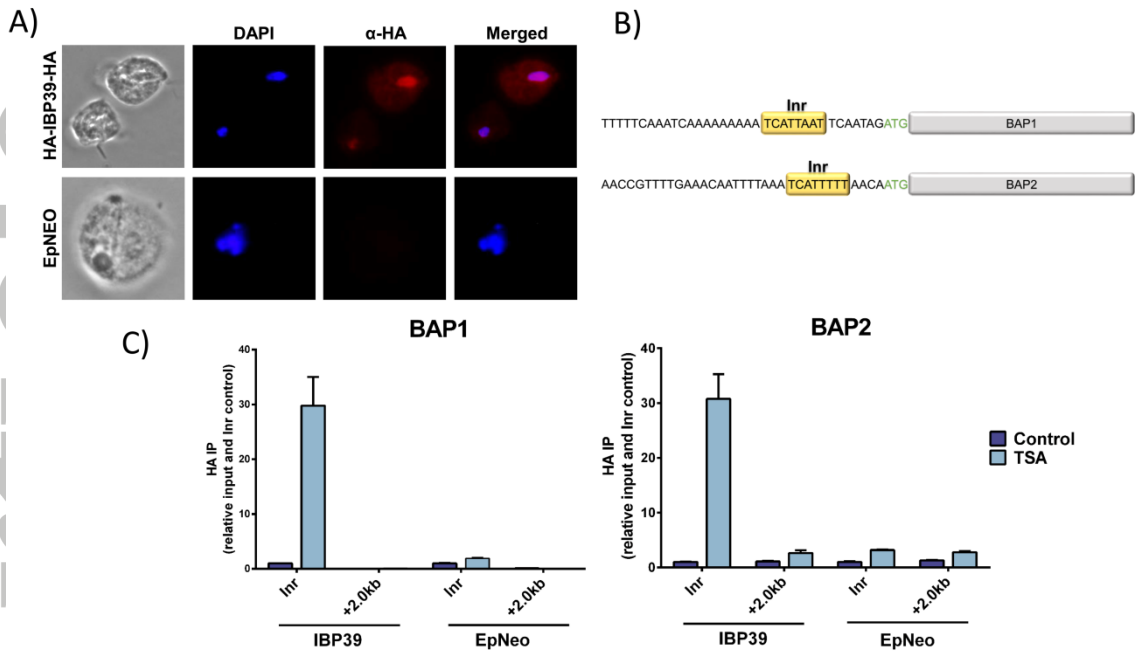


**Fig 6.** ***Histone acetylation modulates IBP39 binding.*** (A) Parasites (G3 strain) transfected with IBP39 HA-tagged on the C- and N-terminal (IBP39-HA) were stained for immunofluorescence microscopy using a mouse anti-HA antibody. The nucleus (blue) was also stained with DAPI. The nuclear localization of IBP39 was observed in more than 50 parasites in two independent transfections. (B). Scheme of 5'UTRs containing the Inr element (highlighted in yellow) in the BAP1 and BAP2 genes. The ATG translation initiation codon is shown in green. (C) Stably-transgenic parasites expressing IBP39-HA were processed for ChIP by using a mouse anti-HA antibody. The promoters of BAP1 and BAP2 genes were tested as they contain an Inr motif. +2 Kbp region of each BAP1 and BAP2 was used as control for non-specific binding as this region lacks the Inr element. Parasites transfected with empty vector (EpNeo) were used as a negative control. Levels of IBP39 binding were normalized to input and G3 untreated control.

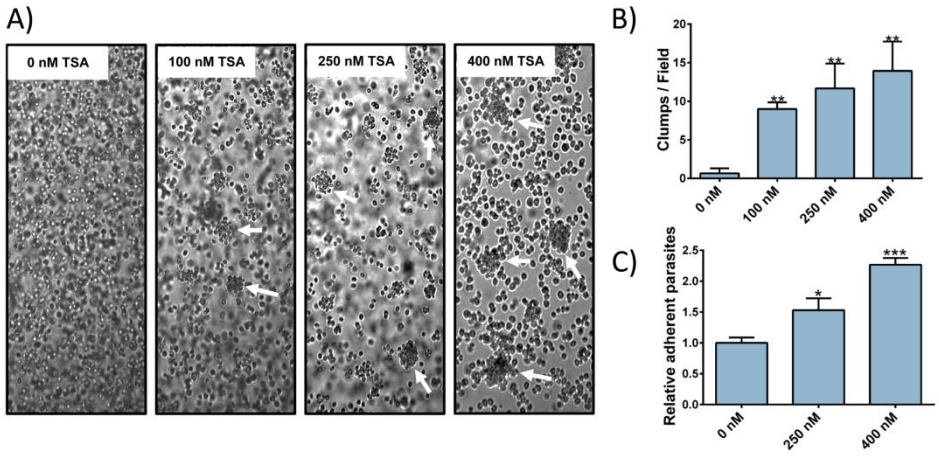


**Fig 7.** ***Histone acetylation regulates parasite aggregation.*** (A) Ability of G3 strain to form clumps after treatment with increasing concentrations of HDAC inhibitor TSA. White arrows indicate a clump. (B) Quantification of clump formation of TSA treated parasites. 30 fields were quantified in three independent experiments. A clump was defined as an aggregate of more than ~5 parasites. Data are expressed as clumps per field ± the standard deviation of the mean. (C) TSA treatment enhances adherence of *T. vaginalis* to host cells. Attachment of G3 parasites treated with different concentration of TSA was assessed and compared with untreated parasites. Data are expressed as -fold increase compared to untreated cells ± the standard deviation of the mean. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.