Dynamic methylation of histone H3K18 in differentiating *Theileria* parasites

Kevin Cheeseman¹,4, Guillaume Jannot¹,4, Nelly Lourenço¹,4, Marie Villares¹,4, Jérémy Berthelet¹,2, Teresa Calegari-Silva¹, Juliette Hamroune³, Franck Letourneur³, Fernando Rodrigues-Lima² & Jonathan B. Weitzman¹

Lysine methylation on histone tails impacts genome regulation and cell fate determination in many developmental processes. Apicomplexa intracellular parasites cause major diseases and they have developed complex life cycles with fine-tuned differentiation events. Yet, apicomplexa genomes have few transcription factors and little is known about their epigenetic control systems. Tick-borne *Theileria* apicomplexa species have relatively small, compact genomes and a remarkable ability to transform leucocytes in their bovine hosts. Here we report enriched H3 lysine 18 monomethylation (H3K18me1) on the gene bodies of repressed genes in *Theileria* macroschizonts. Differentiation to merozoites (merogony) leads to decreased H3K18me1 in parasite nuclei. Pharmacological manipulation of H3K18 acetylation or methylation impacted parasite differentiation and expression of stage-specific genes. Finally, we identify a parasite SET-domain methyltransferase (TaSETup1) that can methylate H3K18 and represses gene expression. Thus, H3K18me1 emerges as an important epigenetic mark which controls gene expression and stage differentiation in *Theileria* parasites.
Our ability to control infectious diseases is improved by our understanding of their complex life cycles and the characterization of pathogen-specific mechanisms that can be targeted by drug strategies. Apicomplexa parasites are a major cause of disease in humans and domesticated animals across the world. For example, *Plasmodium* species cause malaria affecting millions of people each year, emphasizing the need for effective drugs targeting parasites. *Cryptosporidium* is a major cause of diarrhoea in developing countries following infection from contaminated water supplies and there is currently no effective drug therapy. *Theileria* species are bovine-specific pathogens that cause diseases with significant economic impact; Tropical Theileriosis kills over a million cattle per year and costs in the hundreds of millions of dollars. The development of new therapeutic strategies is challenging, as Apicomplexa are eukaryotic cells and share many metabolic pathways with their host animals.

Of all the apicomplexa parasites, *Theileria* is the only eukaryote known to transform its host cell and constitutes a unique model system to explore parasite–host interactions and microbial tumorigenesis. Two *Theileria* species, *T. parva* and *T. annulata* are bovine-specific pathogens that cause severe disease following tick transmission. Infection by these *Theileria* species causes a lymphoproliferative disease in cows with clinical features similar to some human leukaemias. *T. annulata* infects mainly bovine B cells and macrophages, whereas *T. parva* infects bovine B and T lymphocytes. *Theileria*-infected cells are transformed and immortalized; they display uncontrolled proliferation in vitro, independent of exogenous growth factors, and increased ability to migrate and form metastases in immunodeficient mice. Interestingly, *Theileria*-dependent transformation is reversible; animals treated with the theilericidal drug Buparvaquone are cured in most cases. When *Theileria*-infected cells are treated in vitro with Buparvaquone, the intracellular parasite diminishes in the host leucocytes, which lose the transformed phenotype, but drug resistance in the field is an emerging concern for disease control. To achieve transformation, the parasite manipulates the host cell signalling pathways that control cell proliferation and survival. Several host signalling pathways have been implicated in *Theileria*-induced transformation including metabolic pathways, c-Jun Nterminal Kinase (JNK) signaling and host nuclear facts, such as c-Myc, E2F and AP-1. A parasite secreted factor, TaPin1, activates host signaling pathways leading to activation of oncogenic host c-Jun and metabolic gene expression. While previous studies have focused on the host signaling pathways activated by intracellular parasites, very little is known about the regulation of the parasite genome and the mechanisms that orchestrate parasite differentiation and its complex life cycle. A previous study monitored gene expression through merogony, the process of parasite differentiation from intracellular schizonts to infectious merozoites, and reported interesting changes in the levels of transcription factors of the AP2 family. However, the relative paucity of transcription factors in *Theileria* genomes suggests that other mechanisms such as epigenetic pathways may also contribute to parasite differentiation.

Many diseases, especially cancer, are linked to epigenetic events that lead to changes in gene expression. Epigenetic changes associated with disease states include DNA methylation and histone modifications such as lysine methylation and acetylation. Epigenetic enzymes have been causally linked to many diseases making them promising targets for drug interventions. Recently novel drugs that inhibit methylation or deacetylation were developed and some obtained FDA approval to treat cancer. Notably, lysine methylation is emerging as a versatile and dynamic post-translational modification (PTMs) that contributes critically to cellular differentiation programmes. The human genome encodes about 50 biochemically validated lysine methyltransferases (KMTs) that ‘write’ the methylation code and 20 lysine demethylases (KDMs) that act as ‘erasers’. Numerous reports of misregulation of KMTs and KDMs in cancer drove an intense search for specific small-molecular inhibitors. Despite these advances, relatively little is known about the role of epigenetic proteins (methylating Writers or Erasers) in infectious diseases or in infection-induced cancers. The postranslational modification of lysine residues in the histone N-terminal tails plays an important role in regulating chromatin structure and gene expression in all eukaryotes, but has not been previously studied in *Theileria* parasites. We hypothesized that epigenetic modifications, particularly lysine methylation of histone tails, could be a feature of parasite differentiation and that the characterization of parasite encoded epigenetic enzymes could be future drug targets for anti-parasite therapies. In this work we describe the role of methylation of histone H3K18 as an important gene regulatory event during the differentiation of *Theileria* parasites and identify the first parasite methyltransferase capable of methylating H3K18.

**Results**

**Parasite histones are methylated at H3K18.** To initiate a study of epigenetic regulation in *Theileria* parasites, we examined parasite histones focusing on H3. Our analysis of the *T. annulata* genome revealed the presence of two genes encoding histone H3 (Supplementary Fig. 1). The sequences of the N-terminal tails, especially the Lysine residues, are particularly well-conserved in the H3 proteins from *T. annulata, T. parva* and mammals (Supplementary Fig. 1). We, therefore, examined histone modifications using a panel of commercial antibodies recognizing different modified lysine residues in H3 tails. Many of the antibodies we tested by immunofluorescence staining showed strong signals in both host and parasite nuclei; these included relatively well-studied marks such as H3K4me3 and H3K36me3 (see below). However, one modification caught our attention: antibodies recognizing mono-methylated H3K18 (H3K18me1) detected *T. annulata* parasite nuclei, but did not stain bovine host nuclei (Fig. 1a, b). We conducted a series of experiments to pursue the specificity of this initial observation. In contrast to H3K18me1, antibodies against acetylated H3K18 (H3K18ac) displayed strong immunofluorescence signals in both host and parasite nuclei in infected and non-infected bovine B cells (Fig. 1a, b). We observed similar parasite-specific staining for H3K18me1, but not for H3K18ac, in *T. annulata*-infected macrophages TaCl2 (Fig. 1c, d) and in lymphocytes infected with related *T. parva* parasites (Supplementary Fig. 2). Further control experiments with three independent antibodies demonstrated the specificity of the antibody for mono-methylated H3K18 residues, with no cross-reaction to other well-studied H3 lysine methylations (Supplementary Fig. 3). Notably, the sequence around the H3K18 residue is extremely similar in parasite or bovine histones (Supplementary Fig. 1). The difference in monoisotopic mass between the Serine-22 and Thrreonine-22 residues is virtually the same as a methyl group (14.01565): so mass spectrometry could not be used effectively to distinguish between parasite and bovine H3K18 modifications. We confirmed the presence of methylated and acetylated H3K18 in TBL3, TpMD409 and TaCl2 parasite-infected cells by immunoblot experiments (Fig. 1e, f and Supplementary Fig. 2b).

Treatment with the theilericidal drug Buparvaquone (Bup), which kills the parasites, reduced H3K18me1 levels in cell lines infected with *T. annulata* or *T. parva* (Fig. 1e, f and Supplementary Fig. 2b). From these experiments, and careful controls, we conclude
that H3K18 mono-methylation appears to be a feature of *Theileria* parasite schizonts.

H3K18me1 is enriched on the gene bodies of repressed genes in *Theileria schizonts*. Acetylation of H3K18 is a well-studied epigenetic mark linked to gene activation\(^\text{28}\), whereas no studies have focused on the functional role of H3K18 methylation in gene regulation. Indeed, no specific gene regulatory functions have been ascribed to H3K18me1. To investigate H3K18 modifications on the *Theileria* genome, we performed chromatin immunoprecipitation followed by sequencing (ChIP-Seq, performed in duplicates, which we subsequently merged upon good replicate correlations, see Supplementary Fig. 5, to generate the figures shown here) with several antibodies and parallel RNASeq transcriptome analysis. Our dual-sequencing in infected TBL3 cells, followed by reading mapping and bioinformatics analysis, allowed us to simultaneously map epigenetic features on both the parasite and the bovine host genomes. Our analysis of the parasite epigenome revealed some patterns previously described on mammalian genomes; namely, promoter regions around the transcriptional start site (TSS) were enriched for ‘activating’ epigenetic marks such as H3K4me3 or H3K18ac in the parasite genome (Fig. 2a). In contrast, H3K18me1 levels were lowest at the TSS and enriched on the gene bodies (Fig. 2a). Analysis of the bovine genome in the same ChIP-Seq experiments showed enrichment for H3K4me3 and H3K18ac on promoter regions, but no H3K18me1 enrichment on gene bodies, consistent with the parasite-specific immunofluorescence staining results (Supplementary Fig. 4a, b). Gene-body enrichment on actively transcribed genes is a feature of H3K36me3 methylation in mammals\(^\text{29}\). We observed gene-body enrichment for H3K36me3 on genes in the parasite genome (Fig. 2e and Supplementary Fig. 4c). We observed a strong correlation between gene promoter H3K4me3 and H3K18ac, and a weaker correlation between H3K18me1 and H3K36me3 in the parasite genome (Supplementary Fig. 5a–c). The H3K18me1 and H3K36me3 profiles are, however, quite distinct; a differential peak-calling approach on these two modifications revealed differences in the number of peaks (1537 for H3K18me1, 3122 for H3K36me3). We performed k-means clustering analysis of the H3K18me1 profiles on the *T. annulata* parasite genome and defined five clusters (Fig. 2b). Cluster I and Cluster V had the lowest levels of H3K18me1, whereas Cluster IV genes (1103 genes) were enriched for gene-body H3K18me1 (Fig. 2c). In contrast, H3K36me3 levels were relatively high across all clusters, except Cluster V. Comparison with our RNA-Seq data revealed that Cluster IV genes were characterized by lower gene expression compared to Clusters I–III (Fig. 2d, e). Notably, Cluster IV genes were particularly enriched for gene-body H3K18me1 which appeared mutually
**Fig. 2** Mono-methylation of H3K18 is distributed on the gene bodies of repressed genes. 

**a** Average occupancy profiles for H3K4me3 (green), H3K18ac (blue), H3K18me1 (red) and H3K36me3 (purple) around transcriptional start sites (TSS) of all *T. annulata* genes. X-axis: genomic coordinates ± 1.5 kb. Y-axis: log2 (ChIP/Input).

**b** Heatmap of occupancy across all *Theileria* genes starting from 500 bp before the TSS to the end of the gene (TES), normalised by length (normalised to input read coverage). Clustering is the result of a 5 k-means clustering on H3K18me1. The H3K18me1-enriched Cluster IV is highlighted in light grey.

**c** Average occupancy profiles per cluster for H3K4me3 (green), H3K18ac (blue), H3K18me1 (red) and H3K36me3 (purple) starting from 500 bp before the TSS to the TES, normalised by length. Cluster IV is highlighted in grey.

**d** Boxplot of gene expression (RPKM) per cluster as defined by the 5 k-means clusterings on H3K18me1. Pairwise-Wilcoxon statistical testing is indicated. Cluster IV is highlighted in grey. Statistical test Dunnett’s multiple comparison test: ****p < 0.0001. A two-sided pairwise Wilcoxon test was performed. Values: ns: p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Minimum and maximum are represented by upper and lower Whiskers, respectively. Interquartile representation includes first and third quartile, centre representing median, test was performed on three RNA-Seq replicates.

**e** Selected genome region depicting epigenetic profiles and gene expression for *Theileria* genes belonging to the four clusters (indicated below). Two genes from cluster IV are highlighted in grey (TA16455, TA16420). H3K4me3 (green), H3K18ac (blue), H3K18me1 (red) and H3K36me3 (purple), Input (grey). The region ranges from gene TA16455 to TA16400, chr1: 2346753–2365563 (*T. annulata* Ankara strain, accessible at https://www.piroplasmadb.org).
exclusive with H3K18ac and correlated with exonic sequences (Fig. 2e and Supplementary Fig. 5d) ($p < 0.0001$). The genes enriched for H3K18me1 do not appear to cluster on the genome or to group in particular chromosomal locations (Supplementary Fig. 6). Methylation of parasite H3K36 has been linked to repression of var genes in Plasmodium parasites30, but we did not observe regions of strikingly high H3K36me3 enrichment in the Theileria genome (Supplementary Fig. 6). Hence, gene-body enrichment of the H3K18me1 modification appears to be correlated with repression of a large number of genes in T. annulata schizonts.

Parasite differentiation is associated with dynamic changes in H3K18 modifications. In other apicomplexan species, dynamic changes in epigenetic histone modifications accompany changes in gene expression throughout the parasite life cycle25,26,31. Important work from the Shiels laboratory began to define changes in gene expression in Theileria parasites during stage differentiation and implied roles for the ApiAP2 factors19. Little is known about the role of epigenetic mechanisms in regulating gene expression during Theileria differentiation. To investigate the dynamics of H3K18 modifications in Theileria parasites, we induced parasite differentiation from schizonts to merozoites (‘merogony’), by the sustained culture of infected macrophages at elevated temperatures32. The induction of merogony was accompanied by a reduction in H3K18me1 staining in some parasite nuclei (Fig. 3a). After 8 days in the culture at 41 °C, we observed large patches of parasite nuclei with reduced H3K18me1 staining, despite robust H3K4me3 staining across all nuclei (Supplementary Figs. 7 and 8). This suggested that H3K18 methylation dynamics might contribute to parasite differentiation. Unfortunately, there are currently no genetic techniques available to modify the Theileria genome, so we are limited to pharmacological intervention. To test H3K18 dynamics experimentally, we treated cells with inhibitors of lysine demethylases (KDMi) or deacetylases (KDACi), some of the many drugs being developed to inhibit epigenetic enzymes23. Treatment with a
demethylase inhibitor led to a 3-fold increase in parasite H3K18me1 levels and a modest decrease in H3K18ac (Fig. 3b). Conversely, treatment with an acetylase inhibitor robustly increased H3K18ac staining in parasites (Fig. 3b). Importantly, treatment with KDMi or KDACi did not affect the levels of parasite staining with antibodies against H3K4me3 or H3K36me3 (Fig. 3b), demonstrating that the inhibitors do not have a general broad effect on histone lysine methylation. With these pharmacological tools in hand, we sought to test whether these drug-induced effects on H3K18 methylation and acetylation could influence parasite differentiation. We speculate that increased H3K18 methylation might block differentiation, or increased acetylation might favour differentiation. We observed that KDMi treatment significantly reduced the extent of merogony and reduced the expression of the differentiation marker TamR1 (Fig. 4a). Conversely, treatment with KDACi enhanced TamR1 expression, with a relatively mild impact on differentiation (Fig. 4a). These results suggested that dynamic changes in the H3K18 methylation/acetylation balance could affect parasite stage differentiation.

Previous microarray analysis identified sets of genes associated with life-stage transitions in *T. annulata*. Comparison with these data revealed that Cluster IV genes, which are enriched for genebody H3K18me1, are over-represented in differentially expressed genes linked to the sporozoite-tomacroschizont and macroschizont-to-merozoite stage transitions (Fig. 4b and Supplementary Fig. 8). Notably, nine genes encoding *Theileria* ApiAP2 genes that could be stage-transition determinants are also in the list of cluster IV genes (Supplementary Table 3). We analysed the overlap between these datasets, focusing on Cluster...
IV genes and correlations with genes reported to be down-regulated in the sporozoite-to-macroschizont (S2M) transition and upregulated in macroschizont-to-merozoite (M2M) transition. We identified 20 candidate genes (that belong to both M2M_up and S2M_down gene sets, which we termed M2M1-M2M20) that are candidates for genes that are repressed by high H3K18me1 in macroschizonts and upregulated upon differentiation (Fig. 4b and Supplementary Table 1). We also performed a Blast2GO re-annotation of the additional 67 genes that are in the M2M_up group and contained in Cluster IV (Supplementary Table 2). Notably, the 87 genes on these lists (which are candidates for increased expression during the transition from macroschizont to merozoite) include several genes with FAINT domains and signal peptides or transmembrane domains that could be involved in host interactions. It is particularly noteworthy that three proteins (TA05870, TA16660 and TA19445) are homologues of rhoptry proteins characterized in Plasmodium or Toxoplasma as functionally linked to invasion and egress (Supplementary Tables 1 and 2). Gene expression analysis showed that 5 of these 20 genes are indeed significantly induced (over 10-fold) in our hands upon merogony culture conditions (Supplementary Fig. 9a). In contrast, control genes (e.g. parasite TaTMJ1) or bovine genes encoding actin or Hsp70 did not significantly vary upon merogony induction (Supplementary Fig. 9b). We tested whether the drugs that affected H3K18 methylation and acetylation levels could impact the expression of the M2M genes and noted a significant tendency to be induced by treatment with KDACi (see examples in Supplementary Fig. 9c). We chose to study the M2M13 gene (TA16660 encoding a homologue of rhoptry neck proteins) as a striking example (Fig. 4c). The M2M13 gene was induced 60-fold upon merogony differentiation compared to cells grown at 37 °C (Fig. 4c). The temperature-induced expression of M2M13 was blunted by treatment with KDmi at 41 °C (Fig. 4c). To test whether these changes in expression were linked to changes in epigenetic effects and chromatin marks, we performed ChIP analysis of the M2M13 gene locus (Fig. 5a). The M2M13 gene has a peak of H3K18Ac in the promoter region and enriched H3K18me1 across the gene body (Fig. 5a). Treatment with KDmi enhanced H3K18me1 on the M2M13 gene body and promoter (Fig. 5b), whereas the KDACi treatment led to increased H3K18ac on the promoter region (Fig. 5c). This pharmacological intervention changed H3K18 modifications that correlated with gene expression and differentiation (Fig. 4e). Thus, the balance between H3K18me1 on gene bodies and H3K18ac on gene promoters could determine the expression of key genes associated with differentiation stage transitions in *Theileria* parasites.

The *Theileria* SET-domain methyltransferase TaSETup1 can methylate H3K18. The methylation of lysine residues is typically performed by methyltransferases with a characteristic SET domain. We mined the *T. annulata* genome for genes encoding SET-domain containing proteins and identified five candidates (termed here TaSETup1–TaSETup5) (Supplementary Fig. 10 and Supplementary Table 4). Our analysis of expression levels showed that only one of these, the uncharacterized protein TaSETup1, was expressed in macroschizonts and significantly silenced upon merogony differentiation (Fig. 6a). We also identified two potential demethylases with JM1 domains, one of which was induced upon merogony (Supplementary Fig. 10a, b). These observations are consistent with a methylation shift during differentiation. We performed phylogenetic analysis of the parasite putative methyltransferases and found that TaSETup1 resembles a family of mammalian methyltransferases called SMYD3–36 proteins that have been implicated in differentiation and cancer (Supplementary Fig. 11). We used knowledge of SMYD protein structure and function to predict residues whose mutation would lead to a catalytically inactive protein (Fig. 6b, c) 34. Modelling on the known structure of the mammalian SMYD3 protein predicted that mutation of the histidine residue at position 206 should inhibit activity. We performed a highly sensitive, in vitro SAM methyltransferase assays with recombinant TaSETup1 or mutant H206F. Recombinant TaSETup1 could methylate peptides corresponding to H3K18 residues to produce H3K18me1 and H3K18me2/3, whereas the H206F mutant was inactive (Fig. 7a, b). Furthermore, the recombinant TaSETup1 protein methylated core histones (Fig. 7a) as well as recombinant histone H3 or human nucleosomes (Supplementary Fig. 12a–c). We compared the activity of TaSETup1 with SMYD3, the most similar mammalian SET-domain enzyme. Recombinant TaSETup1 could methylate H3K18me1 on recombinant H3, poly nucleosomes or core histone isolated from HEK cells (Supplementary Fig. 13a). In contrast, the related SMYD3 enzyme failed to methylate any of these substrates on K18. But the SMYD3 protein could methylate its preferred substrate MAPK3239 in an in vitro SAM assay (Supplementary Fig. 13b), whereas TaSETup1 could not. Finally, we performed mass spectrometry analysis of histone peptides methylated by the recombinant TaSETup1 enzyme and demonstrated that the parasite enzyme catalyzed monomethylation of the lysine residue corresponding to H3K18 (Supplementary Fig. 14). Thus,
these two highly related methyltransferases exhibit a degree of specificity for different target substrates. No human methyltransferase has been demonstrated to methylate H3K18.

The genetic tools do not currently exist to test TaSETup1 function in live parasites by mutational analysis or knockdown strategies. We attempted to make antibodies that specifically recognized the TaSETup1 protein. Although we obtained antisera that recognized the immunizing antigen and recombinant TaSETup1 protein, these antibodies failed to produce satisfying results in immunoblot or immunofluorescence analysis. To circumvent these constraints, we tested the functional capacity of TaSETup1 to repress gene expression in a heterologous system as H3K18me1 was enriched on the gene bodies of repressed genes. We fused the parasite gene to a GAL4 protein and tested its repressive activity in the T-REx system in which HEK293 T-REx cells contain a stably integrated luciferase (Luc) reporter gene under the control of the thymidine kinase (TK) basal promoter with five GAL4 UAS (Supplementary Fig. 15). Tetracycline-induction led to a significant decrease in luciferase expression linked to TaSETup1, which was lost when the mutant H206F was used (Fig. 7c and Supplementary Fig. 15). Additional experiments using flow cytometry sorting (FACS) of low and high H3K18me1 populations during merogony supported our conclusions that parasite differentiation is accompanied by dynamic changes in histone methylation and changes in TaSETup1 expression (Supplementary Fig. 16).

**Discussion**

The role of epigenetic mechanisms and histone post-translational modifications in regulating gene transcription and cellular differentiation is well-established for a broad range of mammalian genomes and model organisms. Indeed, the enzymes responsible for methylating or acetylating lysine residues in histone tails are identified as targets for drug therapies in a wide range of human pathologies including cancer. However, relatively little is known about how parasite genomes are regulated and the mechanisms controlling differentiation. The paucity of transcription factor characterization in the apicomplexan genomes, suggests that epigenetic mechanisms could play key regulatory roles.

This study presents the first insights into how dynamic changes in epigenetic marks might regulate gene expression and stage differentiation in *Theileria* parasites. We report the first characterization of mono-methylation of the H3K18 residue in parasite histone tails. Our experimental evidence includes immunofluorescence staining of individual cells and immunoblot analysis with three independent and specific antibodies, reinforced by flow cytometry experiments and mass spectrometry analysis with a candidate recombinant methyltransferase enzyme. The H3K18me1 was observed in B cells and macrophages infected with either *T. annulata* or *T. parva* parasites and decreased upon treatment with the thericidal Buparvaquone drug. These results suggest that H3K18 methylation is a general marker of *Theileria* schizonts. Future experiments with primary clinical
samples from infected cows will be interesting to test when the H3K18me1 appears during the parasite life cycle. Our experiments with parasite differentiation towards merozoites suggest that H3K18me1 may prevent merogony and advancement through the parasite life cycle. Our results are consistent with a model in which the repressive function of H3K18me1 is lost on some loci during the differentiation to merozoites and that this allows the upregulation of a subclass of merogony-associated genes. Notably, the 87 proteins in the M2M_up:Cluster IV group include several potential secreted proteins (including Tpr and S genes). Therefore, the 87 proteins in the M2M_up:Cluster IV group likely reflect an upregulation of H3K18me1 silencing the expression of these genes during the transition from macroschizont to merogony. This observation supports the hypothesis that H3K18me1 may play a role in regulating gene expression during the parasite life cycle.

In addition to the novel H3K18me1 epigenetic mark, we identified TaSETup1 as the first SET-domain enzyme capable of H3K18 mono-methylation and the first characterized epigenetic actor in Theileria genomes. As there are only five SET-domain proteins in Theileria genomes, it is likely that parasite methyltransferases are multifunctional, with diverse substrate specificities. Furthermore, there is an emerging interest in non-histone targets and TaSETup1 homologues in other apicomplexa species that may have non-histone substrates. Interestingly, the Toxoplasma homologue TgAKMT is a cytoplasmic protein important for parasite motility and the Plasmodium homologue PfSET7 can methylate H3K4 and H3K9, but also has cytoplasmic functions. Nonetheless, H3K18 methylation has been detected in these parasites and these enzymes could have both cytoplasmic and nuclear functions depending on the parasite life stage. Indeed, TaSETup1 resembles SMYD proteins that have both cytoplasmic and nuclear targets and functions. Studies of parasite epigenetics shed new light on gene regulation mechanisms; another example is the recent discovery of mono-methylated H4K3147 in Plasmodium genomes, it is likely that parasite methyltransferases could have both cytoplasmic and nuclear functions depending on the parasite life stage. Indeed, TaSETup1 resembles SMYD proteins that have both cytoplasmic and nuclear targets and functions. Studies of parasite epigenetics shed new light on gene regulation mechanisms; another example is the recent discovery of mono-methylated H4K3147 in Plasmodium parasites. Methyltransferases represent versatile regulatory enzymes and the drugs being developed to block methylation in cancers could be repurposed for infectious diseases. The study of epigenetic regulation in Theileria is likely to be rich and fruitful and our results already suggest that targeting the methylation machinery to block merogony could be a promising therapeutic strategy.

**Methods**

**Cell culture and treatment.** All bovine cell lines were previously documented: TBL3 cells were derived from in vitro infection of the spontaneous bovine B lymphosarcoma cell line, BL3, with Hissar stock of *T. annulata*. TaC12 is a line of *T. annulata*-infected bovine macrophages. The TpMD409 lymphocyte cell line is
infected with *T. parva*. Cells were cultured in RPMI 1640 (Gibco-BRL), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 4 mM l-glutamine, 25 mM HEPES, 10 mM β-mercaptoethanol and 100 mg/ml of penicillin/streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. HEK 293T-RexLuc cells were grown in DMEM supplemented with 10% FBS and 100 mg/ml of penicillin/streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. The anti-parasite drug Buparvaquone (BW720c) was used at 50 ng/ml for 48 h (Chemos GmbH, ref: 88426-33-9). The KDM inhibitor (KDMi) is the well-characterized, broad-spectrum lysine demethylase inhibitor tranylcypromine (TCP) (a generous gift from A. Mai, Sapienza University, Rome) used at 1 µM, 48 h and the KDAC inhibitor (KDACi) 24 h at 40 nM (compound FR235222 from MA. Hakimi, IAB, Grenoble, France).

**Merogony induction.** Macrogamont-infected TaC12 cells were induced to differentiate to merogony by increasing the culture temperature to 41 °C. Cells were passaged each time they reached confluence and 2 x 10⁶ cells collected at day 0 (macrogamont stage) and day 8 (merogony stage) for RNA extraction and 4 x 10³ cells per immunofluorescence at the same time-points.

**Immunofluorescence analysis.** Cultured *T. annulata*-infected macrophages (TaC12) or B cells (TBL3) were washed with PBS containing 1 mM EDTA and 3 x 10⁶ cells per slide were centrifuged with Cytospin (10 min at 277×g) to adhere to the slide. Cells were fixed in 3.7% paraformaldehyde for 15 min and subsequently permeabilised in 0.2% Triton X-100 (prepared in PBS) for 10 min. Fixation, permeabilisation and all the following steps were carried out at room-temperature.

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**Fig. 7 The *Theileria* TaSETup1 can methylate H3K18 and repress gene expression.**

**a** Methyltransferase assay of recombinant TaSETup1 (wild-type WT or mutant H206F) incubated with or without SAM and core histones followed by immuno-dot-blot detection with H3K18me1 antibody. The quantification results were normalized to H3. This result is representative of three independent experiments showing similar outcomes.

**b** Assay of TaSETup1 methyltransferase activity by quantification of fluorescent H3K18 peptide (substrate S and methylated product P). Chromatograms show mono-methylated (K18me1) and di/tri methylated (K18me2/3) product in presence or absence of SAM for WT and mutant enzymes. Inset: Integration of the peak (AUC area under the curve).

**c** Recruitment of GAL4-TaSetup1 (but not the GAL-TaSETip1 H206F mutant) caused repression of the Luciferase transgene in T-Rex cells in the presence Tetracycline (48 h). The % Luciferase activity represents the mean of n = 3 biologically independent experiments. Error bars represents mean values ± SD.

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| Retention time (min) | TaSETup1 WT | SAM | + SAM | TaSETup1 H206F | SAM | + SAM |
|----------------------|-------------|-----|-------|----------------|-----|-------|
| 0                    | 0           | 0   | 0     | 0              | 0   | 0     |
| 5                    | 5           | 5   | 5     | 5              | 5   | 5     |
| 10                   | 10          | 10  | 10    | 10             | 10  | 10    |
| 15                   | 15          | 15  | 15    | 15             | 15  | 15    |

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antibodies were diluted in PBS and incubated for 1 h at the following dilutions: rabbit anti-H3K18me1 (ab177253, Abcam), 1:5000; rabbit anti-H3K18ac (9675S, Cell signaling) 1:5000; rabbit anti-H3K4me2 (pAb-003-050, Diagenode) 1:200. Cells were subsequently washed three times with PBS and incubated with secondary antibody for 30 min at the following dilutions: Alexa594-conjugated donkey anti-rabbit antibody, 1:1000. Cells were washed three times with PBS and finally, mounted on coverslips adding Prolong Diamond Antifade Mountant with DAPI. Samples were analysed using a Leica DMi 6000 epifluorescence microscope. Images were generated and processed using Metamorph and Image software.

For H3K18ac and H3K18me1 intensity quantification in the parasite, for each cell, we calculated the mean intensity in the entire parasite using ICY software, divided by the number of parasite nuclei per host cell. The counting of cells in the macroschizont or merogony stage was done using ImageJ. We defined a threshold for the Schizont/Merogony cycle stage at 80 parasites per cell.

Protein extraction and Western blot analysis. For all the cell lines, 2 × 10⁶ cells were collected and histone extraction was performed using the Abcam Kit protocol. Histones were resolved on NuPage 4–12% acrylamide gradient SDS–PAGE gel (Invitrogen) and transferred onto nitrocellulose membranes in Tris–glycine transfer buffer. Membranes were blocked and incubated overnight at 4 °C with the primary antibodies: H3K18me1 (ab177253) 1:20,000; H3K18ac (ab1191) 1:2000; H3 (ab1791) 1:10,000. Membranes were incubated with the appropriate secondary antibody coupled to horseradish peroxidase (HRP), revealed using West Dura kit and the Licit detection system.

Luciferase reporter activity. HEK 293T-ResLuc cells were grown in DMEM supplemented with FBS, 4% (v/v) FBS, and transfected with the pcDNA4-To-Ga4-G9a, pcDNA4-To-Ga4-TaSETup1, pcDNA4-To-Ga4-Pr5r7, and pcDNA4-To-Ga4-TaSETup1-H207F plasmids using lipofectamine 2000. The proteins were induced 5 h post-transfection with Tetracycline (final concentration of 1 µg/ml). Transfection efficiencies were normalized to Renilla activity by co-transfection of a pRL-TK Renilla reporter plasmid at 150 ng. Luciferase assays were performed 36 h post-induction using the Dual-Glo Luciferase assay system (Promega) in a microplate luminometer. The percentage of luciferase activity was represented as the ratio Firefly/Renilla luminescence, compared with the non-induced transfected cells.

Plasmids and transfection. pcDNA4-To-Ga4-G9a and pcDNA4-To-Ga4-Pr5r7 were a gift from S. Ait-Si-Ali. pcDNA4-To-Ga4-TaSETup1 and pcDNA4-To-Ga4-TaSETup1-H207F were generated from pcDNA4-To-Ga4-G9a cloning TaSETup1 cDNA from Theileria infected macrophages RNA, with restriction enzymes EcoRI and NotI. Each plasmid was transfected in HEK 293T-ResLuc cells with Lipofectamine 2000 in a concentration gradient (0.5, 1 and 2 µg). pRL-TK Renilla reporter plasmid was co-transfected at 150 ng.

RNA extraction and RT-qPCR. For all cell conditions, total RNA was extracted using a Nucleospin RNA extraction kit (Macherey-Nagel) following the manufacturer’s protocol. 1 µg of total RNA was reverse transcribed with Superscript III polymerase (ThermoFisher). The open reading frame of TaSETup1 (TA06820, piroplasmaDB) (refseq_948938) was PCR amplified with Q5 high fidelity DNA polymerase (Edwards). PCR products were purified with Q5 high fidelity DNA polymerase (Edwards). PCR products were purified with Q5 high fidelity DNA polymerase (Edwards). PCR products were amplified with primers designed with Primer3 (v3.3) and cloned by BP recombination reaction into entry clone pCDNA4-TO-Gal4-G9a and pcDNA4-TO-Gal4-TaSETup1. Restriction enzymes, EcoRI and NotI, were digested with Q5 high fidelity DNA polymerase (Edwards). PCR products were purified with Q5 high fidelity DNA polymerase (Edwards).

Phylogenetic analysis. SET domain proteins were retrieved from interProDB662 using the SET domain identifier IPR0014. Homo sapiens SET proteins were further curated to include only some representatives of the major SET families. Proteins were aligned using mafft53 v7.245 with option --localpair and--maxiterate 1000 --ep 0. Alignment was examined using Jalview54 v2.10.5 and K-means clustering on H3K18me1 ChIPseq analysis was performed using the computeMatrix and plotHeatmap tools. Circos plot of CHIP-seq and RNA-seq experiment was done using circie31 (v0.4.3).

Reannotation of parasite genes. T. annulata protein sequences were submitted to the blast2GO5 pipeline for annotation using standard parameters.

Phenylogenic analysis. SET domain proteins were retrieved from interProDB662 using the SET domain identifier IPR0014. Homo sapiens SET proteins were further curated to include only some representatives of the major SET families. Proteins were aligned using mafft53 v7.245 with option --localpair and--maxiterate 1000 --ep 0. Alignment was examined using Jalview54 v2.10.5 and phylogenetic tree inferred with Ultra-Fast bootstrap and alrt branch support using iqtree55–57 v1.5.5 with options --m 3072 -h 1000 –x 1000. FigTree v1.4.3 was used for tree annotation. Computations were performed using Docker containers available at https://hub.docker.com/u/paigepigenetics/.

Reannotation of parasite genes. T. annulata protein sequences were submitted to the blast2GO5 pipeline for annotation using standard parameters.

Cloning and site-directed mutagenesis. Total cellular RNA from infected macrophages (TaCi2) was converted to cDNA using Phusion high fidelity DNA polymerase (ThermoFisher). The open reading frame of TaSETup1 (TA06820, proplasmodaDB) (refseq_948938) was PCR amplified with Q5 high fidelity DNA polymerase (NEB) and cloned by BP recombination reaction into entry clone pDONR207 using GATEWAY cloning technology. The positive entry clone containing TaSETup1 was shuttled into pDEST17 His Tag Expression vector by LR recombination reaction using GATEWAY cloning technology. For site-directed mutagenesis of TaSETup1, Histidine 206 residue was mutated into phenylalanine using Phusion Site-Directed Mutagenesis Kit (ThermoFisher). The resulting positive clone was transformed to competent E. coli host HI-control BL21 (DE3) cells for protein expression and purification.

Protein expression and purification. A single colony of pDEST17 TaSETup1 WT, or H206F, was inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin. The overnight culture was transferred to 1 l of fresh medium and was grown at 37 °C until OD value of 0.7 at 600 nm was reached. Isopropyl β-D-thiogalacto- pyranoside (IPTG) was added to a final concentration of 0.5 µM and grown overnight at 16 °C. Cells were harvested by centrifugation at 2400g for 20 min and the pellet re-suspended for 30 min at 0 °C under agitation in cold lysis buffer.
(300 mM NaCl in PBS 1×, pH 8, triton 1%, lysoyzme 1 mg/ml, 10 mM imidazole, cOpteMed Protease Inhibitor Cocktail). The cells were disrupted by sonication on ice and clarified by centrifugation at 10,000 × g for 30 min at 4°C. Proteins carrying the histidine tag were purified using His-Select Nickel Affinity Gel. Briefly, the clarified lysate was incubated with affinity beads for 3 h at 4°C under agitation and then transferred to a chromatography column. After extensive washing, the histidine-containing protein was eluted from the column using 5 column volumes of buffer (300 mM NaCl in PBS 1×, pH 8, 5 mM Imidazole). Samples were subjected to buffer exchange into the low salt buffer (Tris–HCl 50 mM, NaCl 50 mM, pH 8) using PD-10 Desalting Columns containing Sephadex G25 resin (GE Healthcare). Desalted samples were concentrated using Amicon Ultra centrifugal filter units (cutoff 10 kDa, EMD Millipore) and subjected to gel filtration using a HiLoad 16/60 Superdex 200 size-exclusion column using an AKTA purifier system (Cytivia). The sample was eluted using the low salt buffer and fractions containing His-tagged protein were pooled, concentrated using Amicon Ultra centrifugal filter units (cutoff 10 kDa) and analysed by SDS–PAGE gel electrophoresis and Coomasie staining.

**RP–UFLC–based separation and quantification of the fluorescein–labelled peptide substrate of TaSeTP1 (FAM-H3K18) and its methylated products (FAM-H3K18me).** A 9- amino–acid peptide derived from the sequence of human histone H3.1 protein and containing the lysine 18 residue was synthesized and conjugated to fluorescein amide (FAM) on its N–terminus and modified by amida- tion (NH2) on its C–terminus (Proteogenix, France). The lysine 14 was mutated to arginine in order to only monitor lys–18 TaSeTP1–dependent methylation. The peptide was as follows: FAM–RPRKQoQLAT–NH2. A mono, di, and tri–methylated different forms of H3K18 (H3K18mon, H3K18dim, H3K18trim) peptide was also synthesized and used as standard. The lysine methyltransferase reaction was carried out overnight at room temperature in methylation buffer (Tris 50 mM pH 8, 50 mM KCl, 300 mM NaCl containing 75 µM of the peptide substrate and enzyme and with or without 200 µM of S–Adenosyl methionine (SAM). Samples containing H3K18 peptide (substrate) and its methylated products (products) were separated by RP–UFLC (Shimadzu) using Kromasil 100–5–C18 column 4.6 × 250 mm, 5 µm particle size at 40 °C. The mobile phase used for the separation consisted of two solvents. Solvent A containing water with 0.1% perchloric acid and Solvent B containing acetoni trile with 0.12% trifluoroacetic acid (TFA). Separation was performed by an isotropic flow as followed: 80% A/20% B, rate of 1 ml/min, time of run = 27 min. H3K18 peptide (substrate) and its methylated products (products) were monitored by the fluorescence emission (λ = 530 nm) after excitation at 490 nm. The peak area was quantified by integration of the peaks and calculated using the area correction curve, employing a calibration curve established with various known concentrations of peptides.

**Histone methyltransferase in vitro assays.** Recombinant histone H3 (NEB), H3 from calf Thymus (Sigma), or core histone purified from chicken erythrocytes (Sigma) (500 ng) were mixed with or without S–adenosyl methionine (100 µM final) in 1 µl MT buffer (10 mM Tris pH 8.0, 20 mM KC1, 5 mM MgCl2, 5% glycerol, 1 mM DTT) in a final volume of 25 µl and incubated at room temperature for 2 h. Samples mixtures were immobilized on nitrocellulose membrane using Bio–Dot microfiltration apparatus (Bio–Rad) and blocked with 5% non–fat milk in PBS–TWEEN for 1 h. Membranes were then incubated with antibodies against H3K18me1 (Abcam #ab177235, Active Motif #51295) and a home–made antibody provided by Jane Mellor's laboratory (1:10,000) or H3K36me3 (Abcam #ab9050) (1:10,000) at 4°C overnight. Membranes were washed three times with PBS–TWEEN for 10 min and incubated with a 1:20,000 dilution of horseradish peroxidase–conjugated anti–rabbit antibody for 1 h at room temperature. Membranes were finally developed with the Pierce ECL Western Blotting Substrate according to the manufacturer's protocol.

**Flow cytometry analysis.** Cells were washed once with PBS and then fixed overnight in 0.5% PFA in PBS at 4 °C. After centrifugation (5 min at 300× g), cells were washed in PBS, resuspended and resuspended in 0.1% Triton–X (5 min) then washed once in FACS Buffer (cold PBS, 20 mM Heps and 0.5% BSA) Then, primary antibodies were added in FACS buffer and incubated 20 min at 4 °C. Cells were washed with FACS buffer and incubated with secondary antibody at the same dilution conditions as immunofluorescence experiments above. Cells were washed again with FACS buffer and resuspended in 200 µl of FACS buffer for flow cyto-metry analysis.

The sequences of all primers and oligonucleotides used in this study are listed in Supplementary Table 5.

**Data availability**

All data generated or analysed during this study are included in this published article. The RNA–Sequencing and ChiP–Seq data have been deposited to the ENA database with the study identifier PRJEB37392. The Zenodo access link is provided (https://doi.org/10.5281/zenodo.3700834) DOI number: https://doi.org/10.5281/zenodo.3700834.

**Conclusion**

This repository provides bigwig files for ChiP and RNA sequencing experiments in *Theileria annulata*. DeepTools generated Coverage files, ReadCount normalised files and SES normalised files are provided. Also provided are genome and annotation files for visualisation with IGV software. The Mass Spectrometry proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024599. Source data are provided with this paper.

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**Characterization of H3K18me1 antibody specificity.** 2 µg of 5–FAM coupled short peptides (Proteogenix) flanking the unmodified, monomethylated, dimethylated, trimethylated or acetylated lysine of interest (H3K4: ARTKQTARRSK, H3K9: RQARTKGGG, H3K14: STGGKAPRR, H3K18: TKAARKSAPAT and H3K36: TGGKVRKPR) were transferred on nitrocellulose membrane using Bio–Dot microfiltration apparatus (Bio–Rad) and blocked with 5% non–fat milk in PBS–TWEEN for 1 h. Membranes were then incubated with antibodies against H3K18me1 (Abcam #ab177235, Active Motif #51295) and a home–made antibody provided by Jane Mellor's laboratory (1:10,000) or H3K36me3 (Abcam #ab9050) (1:10,000) at 4°C overnight. Membranes were washed three times with PBS–TWEEN for 10 min and incubated with a 1:20,000 dilution of horseradish peroxidase–conjugated anti–rabbit antibody for 1 h at room temperature. Membranes were finally developed with the Pierce ECL Western Blotting Substrate according to the manufacturer's protocol.

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**Author contributions**

J.B.W. developed the conceptual framework, sought funding, wrote the manuscript and provided overall supervision. K.C., G.J., N.L., M.V., T.C.S., J.B., J.H., designed and performed experiments, analysed the data and helped write the paper. F.L. and F.R.L. provided supervision and expertise. K.C., G.J., N.L. and M.V. made equal contributions and are listed in alphabetical order.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence** and requests for materials should be addressed to J.B.W.

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