Microreview

Chromatin modifications: implications in the regulation of gene expression in *Toxoplasma gondii*

Alexandre Bougdour, Laurence Braun, Dominique Cannella and Mohamed-Ali Hakimi*

Laboratoire Adaptation et Pathogénie des Micro-organismes, CNRS UMR 5163 – ATIP Group, Université Joseph Fourier, BP 170, F-38042 Grenoble cedex 9, France.

Summary

The apicomplexan *Toxoplasma gondii* completes its life cycle by successive processes of parasite differentiation that rely on a tight control of gene expression to ensure appropriate protein profiles on time. During the last 5 years, several groups have pioneered this field of investigation, suggesting that epigenetics could play an important role in the control of parasite gene expression. Histone modifications serve as an effective way to regulate gene transcription but they do not operate alone; rather, they act in concert with other putative epigenetic information carriers (histone variants, small RNAs) and DNA sequence-specific transcription factors to modulate the higher-order structure of the chromatin fibre and govern the on-time recruitment of the transcriptional machinery to specific genes. Regarding the ‘histone code’ hypothesis, the parasite is endowed with a rich repertoire of histone-modifying enzymes catalysing site-selective modifications, which are subsequently interpreted by effector proteins that recognize specific covalent marks. Still, several peculiarities seem unique to *T. gondii*. This review is a synthesis of the current knowledge of how epigenetics contribute to the control of gene expression in *T. gondii* and, likely, other Apicomplexa.

Introduction

Apicomplexa protozoans form a phylum of obligatory intracellular parasites that contains important human pathogens, including the agents of malaria (*Plasmodium* sp.) and of toxoplasmosis (*Toxoplasma gondii*). Toxoplasma is the most widespread apicomplexan parasite, present virtually everywhere on earth. Although usually causing only mild symptoms in the adult, it can cause severe and life-threatening diseases in developing fetuses and in immunocompromised individuals, especially AIDS patients. *T. gondii* has a heteroxenous life cycle relying on the differentiation into several morphologically distinct forms, including both sexual and asexual stages. *T. gondii* displays an unusual population structure consisting of three predominant clonal lineages, designated types I, II and III, that are abundant in Europe and North America (Sibley and Ajioka, 2008). Although the genetic diversity between these lineages appears to be low, there are obvious phenotypic differences in virulence, migration, growth rate, and the ability to convert from tachyzoite to the cyst forming bradyzoite stage (Behnke et al., 2008; Blader and Saeij, 2009). Changes in gene expression are expected (i) as parasites progress through the cell cycle, (ii) as parasites differentiate in specific stages and (iii) as parasites are exposed to the host immune system during infection. Transcriptional profiling across asexual development of *P. falciparum* (Bozdech et al., 2003; Le roch et al., 2003) and *T. gondii* (Radke et al., 2005) suggests a model where a cascade of gene expression results in a ‘just-in-time’ production of products only when needed. How these changes are regulated at the molecular level remains, to a large extent, unknown.

An unexpected feature is the apparent lack in Apicomplexa of large families of recognizable specific transcription factors (TFs) operating in other eukaryotes (Iyer et al., 2008). This observation is paradoxical given the complex life cycle of these parasites, which certainly require a tight regulation of gene expression. What we and others have initially proposed was that although many phenotypic differences in *T. gondii* are genetically encoded, epigenetic control could be part of the parasite developmental programs and adjustments to fluctuant environment (Sullivan et al., 2008).
and Hakimi, 2006; Hakimi and Deitsch, 2007). Epigenetic regulation, which includes potentially heritable changes in gene expression that do not involve changes in DNA sequence, provides a mechanism by which an organism can maintain a type of short-term memory of its most recent environment, allowing it to respond quickly to changing conditions. The initial hypothesis had to be re-assessed in light of the recent discovery in Apicomplexa of an expanded family of plant-like TFs harbouring APETALA2 (AP2)-like domains (Balaji et al., 2005; Iyer et al., 2008).

The packaging of DNA into chromatin is recognized to be a major mechanism by which the access of genomic DNA is restricted and regulated. The chromatin is constantly remodelled to provide either a more accessible and transcriptionally active structure termed ‘euchromatin’ or a more compact, less accessible and transcriptionally silent structure termed ‘heterochromatin’ (Li et al., 2007). A number of molecular processes play a role in the dynamic transition between these two transcriptionally distinct states, including nucleosome assembly and remodelling, histone replacement, reversible modification of histones, methylation of DNA and changes in sub-nuclear localization of chromatin (Goldberg et al., 2007; Kouzarides, 2007; Li et al., 2007).

DNA methylation is a highly stable silencing mark that extends over long chromosomal regions leading to ‘memorised’ states of gene expression that may be inherited from generation to generation. Post-translational modifications (PTMs) of histone subunits, such as acetylation, may be more labile and mediate regulation of gene expression over shorter-term periods. The functional consequences of histone PTMs can be direct, causing structural changes to chromatin and thereby affecting the DNA accessibility, or indirect, acting through the recruitment of effecter proteins (Goldberg et al., 2007; Kouzarides 2007). Various models such as the charge-neutralization model (Roth and Allis, 1992; Shogren-Knaak et al., 2006) and the histone code (Strahl and Allis, 2000; Turner, 2000), as well as a signalling pathway model (Schreiber and Bernstein, 2002), have been proposed to explain the role of histone modifications in transcription. The charge neutralization model, in which histone PTMs directly affect chromatin compaction, include phosphorylation or acetylation on core histones that serves to attenuate the favourable coulombic interactions between basic histone proteins and the negative charge of the DNA (Shogren-Knaak et al., 2006). The ‘histone code’ hypothesis predicts that diverse covalent modifications within the highly accessible histone tails are read by effector molecules, which in turn generate distinct outcomes (Strahl and Allis, 2000; Turner, 2000). In this model, PTMs work in concert, and the cross-talk between different modifications determines the final biological read-out. In this context, some modifications can influence others, and it appears that specific combinations of these modifications can form a complex and dynamic code.

As T. gondii does not appear to have detectable DNA cytosine methylation (Gisot et al., 2008), remodelling of the chromatin structure particularly through PTMs of histones is potentially a major process that co-ordinates regulation of gene expression. For this reason, histone modifications have rapidly moved to the forefront of gene regulation research in many protozoan parasites. Histone PTMs serve as an effective way to regulate parasite gene expression but they do not operate alone; rather, they appear to act in concert with other putative epigenetic information carriers (histone variants, small RNAs) and DNA sequence-specific TFs (AP2-like or ApiAP2) to modulate the higher-order structure of the DNA fibre and govern the on-time recruitment of the transcriptional machinery to specific genes. In this review, we focus our remarks on specific examples of how PTMs are written, erased and interpreted in ways that they might contribute to gene expression regulation in T. gondii and, likely, other Apicomplexa. Several broad questions can be posed at this occasion. How is the parasite epigenetic dialect read by chromatin tethering modules in T. gondii and how is the epigenetic information transduced into a cellular response? Do epigenetic modifications influence the manner TFs are associated with DNA? Is the reading critical for re-programming the genome during stage conversion?

A large repertoire of PTMs is present in T. gondii

The functional epigenetic landscape is much more complex than previously thought in Apicomplexa. We and others have begun our investigations by looking at the evolutionary conservation of histone modifications. Our goal in this review is not to provide a compilation of them but rather to illustrate general principles as they might apply to the life cycle, by specifically highlighting distinct examples to discuss further. Unbiased mass spectrometry (Miao et al., 2006; Trelle et al., 2009) and antibody based detection techniques (Saksouk et al., 2005; Sautel et al., 2007; Issar et al., 2009) have led to the description of the repertoire of the most prevalent histone PTMs of the core histones of P. falciparum and T. gondii (updated repertoire in Fig. 1). When compared with other model organisms, PTMs are more conserved than previously thought in Apicomplexa although there were substantial differences in their abundances. The most remarkable finding was that H4K16 is not the major acetylation site in P. falciparum histone H4, which contrasts with what is observed in most other organisms (Zhang et al., 2002; Smith et al., 2003). Instead, acetylation of H4K8 and H4K12 was frequently observed in both P. falciparum (Trelle et al., 2009) and T. gondii (M.A.H., unpubl. data). In general, parasites
appear to harbour more activation (H3K4me or H3K9ac) than silencing marks (H3K9me) when compared with human cells (Trelle et al., 2009). The histone variants are no exception to this rule, since they are extensively acetylated in their tails probably to maintain open chromatin structure (Fig. 1) (Trelle et al., 2009). This observation mirrors the studies on Saccharomyces cerevisiae and Tetrahymena thermophila that exhibit more modifications associated with transcriptional activation than repressive marks, whereas the reverse situation is observed in mammals (Garcia et al., 2007). This correlates quite well with the fact that the majority of the P. falciparum genome is transcriptionally competent (Bozdech et al., 2003; Le Roch et al., 2003), whereas more than 60% of the mammalian genome is permanently silenced (Jenuwein et al., 1998; Whitfield et al., 2002).

Fig. 1. Repertoire of the most prevalent histone PTMs in the core histone subunits of T. gondii and P. falciparum identified by mass spectrometry and/or antibody-based detection techniques. The predicted enzymes in charge of the writing or the erasing of the PTMs are indicated above (or below) the modified residue: TgPRMT1 (TGME49-019520), TgCARM1 (TGME49-094270), TgPRMT5 (TGME49-015560), TgJMJD6 (TGME49-061260), TgJARID1 (TGME49-107010), LSD1 (TGME49-075420/TGME49-042420), TgSET1 (TGME49-026810), TgSET2 (TGME49-057770), TgSET3/SUV3-9 (TGME49-05970), TgSET8 (TGME49-011730), TgGCN5-A (TGME49-054550), TgGCN5-B (TGME49-043440), TgSIR2 (TGME49-042700), and TgHDAC3 (TGME49-027290).
With regard to histone modifications, few divergences were observed so far between T. gondii and P. falciparum. The one known exception is the serine 10 phosphorylation of histone H3, which is involved in both transcription and chromosome condensation during mitosis in eukaryotic cells (Prigent and Dimitrov, 2003). While in T. gondii H3S10 is phosphorylated (H3S10ph) prior to mitosis in tachyzoites (Sautel et al., 2007), the mark is apparently absent or too low to be detected in P. falciparum (Trelle et al., 2009). This discrepancy is interesting and calls for further investigation.

Overview of the histone modifying enzymes

Qualitative examination of Apicomplexa genomes reveals a rich but still largely unexplored repertoire of ATP-dependent remodelers and histone-modifying enzymes that move, replace or decorate histones (see recent reviews by Sullivan and Hakimi, 2006; Hakimi and Deitsch, 2007; Horrocks et al., 2009). What did we learn so far? First, these parasites possess a sophisticated capacity to modify histones, rivaling the system observed in higher eukaryotic cells. For instance, there was significant duplication and divergence in the Apicomplexa lineage of the lysine methyltransferases (KMT) harbouring a conserved Suv(39)-E(z)-TRX (SET) catalytic domain (Sautel et al., 2007; 2009). Unexpectedly, we show that the phylum harbours genes coding for enzymes phylogenetically and structurally related to Set8, a family of proteins previously thought to be restricted to metazoans (Sautel et al., 2007). Remarkably, biochemical and structural modelling analyses demonstrated that T. gondii TgSet8 can transfer mono-, di- and trimethyl H4K20 sequentially, whereas its human counterpart is exclusively found as a monomethylase (Sautel et al., 2007). Beyond lysine methylation, a more extensive repertoire of arginine methylation machinery is present in T. gondii compared with yeast and C. elegans; each possessing a unique PRMT related enzyme (Saksouk et al., 2005).

In general, duplication and divergence of histone modifiers is more pronounced in T. gondii than in P. falciparum. Notably, T. gondii appears to be unique inside the phylum in harbouring more than one GCN5 family histone acetyl-transferase (HAT) (Bhatti et al., 2006). Duplication does not mean redundancy since TgGCN5-B acetylates H3K9, K14 and K18, the expected substrate profile for archetypical GCN5 HATs (Bhatti et al., 2006), while TgGCN5-A exhibits a strong tendency to acetylate in vitro H3K18 (Saksouk et al., 2005). Likewise, the JmjC (Jumonji)-containing domain demethylases were expanded in T. gondii with at least seven members while Plasmodium species have only two (Fig. 2A). Beyond the two putative enzymes that are homologous to JARID-like H3K4 and JMJD1-like H3K9 demethylases, T. gondii genome harbours two orthologous genes that belong to JMJD6 family of proteins known for demethylating H3R2 and H4R3 (Fig. 2A) (Chang et al., 2007). The remarkable number of JmjC-containing demethylases present in the T. gondii genome could be correlated to the striking expansion of the methylases targeting lysine (19 versus 9 in Plasmodium genus) or arginine (5 versus 3 in Plasmodium genus) residues (Saksouk et al., 2005; Sautel et al., 2007; Cui et al., 2008). In terms of diversity, the chromatin-modifying apparatus of T. gondii goes beyond our expectations and this was consistent with the initial hypothesis that the apparent lack of traditional TFs may be somehow compensated at an epigenetic level.

Genomic profiles of histone modifications

Genome-wide mapping of epigenetic marks, binding sites for TFs, core transcriptional machinery and other DNA-binding proteins is critical for deciphering the gene regulatory networks in T. gondii. Chromatin immunoprecipitation (ChIP) has become a powerful tool for understanding transcriptional cascades and interpreting the information encoded in chromatin (Schones and Zhao, 2008). Chromatin immunoprecipitation followed by microarray analysis (ChIP–chip) has revealed a genome-scale view of the specific protein–DNA interactions in T. gondii and P. falciparum. Studies mapping specific PTMs over the genome of T. gondii and P. falciparum suggest that the interplay between chromatin and transcription is dynamic and more complex than previously appreciated (Gissot et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009). Genome-wide data obtained from T. gondii revealed that H3K9me2 and H3K9me3 repressive marks tend to spread over large regions of heterochromatin, particularly near the centromeres (C.F. Brooks, M. Gissot, K. Kim and B. Striepen, unpubl. work; H3K9me2 data were deposited at http://www.ToxoDB.org). In contrast, in P. falciparum H3K9me3 exhibits a restricted pattern at subtelomeric regions with no enrichment at the pericentromeric chromatin like in T. gondii (Lopez-Rubio et al., 2009). TgSet8 that methylates H4K20me1,3 appears also to associate with the T. gondii centromeres themselves (Sautel et al., 2007 and M.A.H., unpubl. data). In P. falciparum, H4K20me3 shows a broad distribution across the genome at the ring stage (Lopez-Rubio et al., 2009) although in early schizont the mark is detected at telomeric foci (Sautel et al., 2007). This discrepancy is interesting and calls for further investigation; it might be expected that H4K20me3 is also differentially redistributed in sporozoite and bradyzoite differentiated T. gondii parasites. Altogether, these data highlight important differences in the utilization of histone silencing marks amongst these two parasites. H3K9me3 has been correlated with gene silencing, but this residue can also be acetylated to generate an
Fig. 2. A. Evolutionary relationships between T. gondii (gene number according to TOXODB 4.3), P. falciparum (MAL8P1.111 and PFF0135w), Arabidopsis thaliana (AT), Homo Sapiens (Hs) and S. pombe (Sp) Jumonji (Jmj) C-domain-containing demethylases. T. gondii genome displays at least five distinct lineages of the JmJ-containing proteins represented as shaded boxes. The unrooted phylogenetic tree was inferred from the JmjC domain alignment. Numbers above the nodes indicate neighbour-joining bootstrap percentages.

B. Protein interaction network with six P. falciparum ApiAP2 transcription factors. The yeast two-hybrid data are available at http://www.plasmodb.org.
activation mark. Unexpectedly, H3K9ac in concert with another activation mark H3K4me3 are spread equally across active and inactive \( P. \) falci-paraum genes at the ring stage, whereas in schizonts they are distinctively enriched at the 5′ end of active genes (Salcedo-Amaya et al., 2009). At silent promoters, the marks are probably maintaining a poised activation state or serving as a memory of previous transcriptional activation as described in human cells (Roh et al., 2006; Guenther et al., 2007). These results contrast deeply with those in \( T. \) gondii tachyzoites where both H3K4me3 and H3K9ac are predominantly enriched at the 5′ end of active genes. In addition, most of the \( T. \) gondii genes harbouring simultaneously H3K9ac and H3K4me3 marks are expressed in all three canonical strains with few exceptions at certain loci where the marks are not associated (Gissot et al., 2007; see genome-wide results at http://www.ToxoDB.org). Remarkably, as for gene silencing there are distinct epigenetic pathways in gene activation in Apicomplexa parasites, tailored to their own needs. The distribution pattern of H3K4me3 and H3K9ac in \( P. \) falci-paraum ring stage versus schizont is one elegant example (Salcedo-Amaya et al., 2009) although more are expected to be found in the future.

Such approaches clearly showed that histone PTMs are sequestered to distinct regions of the genome. However, the genome coverage in ChIP-chip is limited by the repertoire of probe sequences fixed on the array. This is particularly important for the analysis of repetitive regions of the genome, which are typically masked out on arrays. ChIP–seq now offers higher resolution, less noise and greater coverage than its array-based predecessor. The next goal lies in the integration of ChIP-seq data with quantitative measurements of transcriptomes by RNA-seq to link regulatory inputs with transcriptional outputs and at term to ultimately reconstruct dynamic gene regulatory networks over the different parasites stages or stress conditions. In addition, histone PTMs patterns could be a useful tool to achieve a more precise annotation of the Apicomplexa genomes. They have been used to identify novel transcriptional units and determine functional transcription start sites in \( T. \) gondii (Gissot et al., 2007).

Interfering with the epigenome to understand gene expression

Our current understanding of histone modifications in these parasites is mostly based on a catalogue of modifications without a clear view of how the chromatin landscape can be translated into specific biological outputs such as transcription. The interest of Duraisingh et al. (2005) in the regulation of var gene expression by the NAD+-dependent HDAC PfSir2 led to the demonstration that this enzyme is essential for repressing a subset of var genes in \( P. \) falci-paraum. Freitas-Junior et al. (2005) showed that the presence of PfSir2 at the level of certain promoters correlates with the histone acetylation status, therefore suggesting that PfSir2, through its HDAC activity, is a direct regulator of var gene transcription and thereby an important regulator of antigenic variation. These data fit well with the model that histone acetylation favours transcription whereas hypoacetylation corresponds to heterochromatic regions. Although it is clear that PfSir2 enzymes (PfSir2A and PfSir2B) control var gene transcription (Tonkin et al., 2009), the mechanisms controlling their activities and recruitment to the chromatin is still obscure.

At term, ChIP analysis should reveal the order in which complexes are recruited, the concurrent chomatrin modifications present, and their resulting marks at both gene and genome-wide scales during transitions from transcriptional repression to activation, to attenuation and back to repression. One way to examine dynamic changes in PTMs patterns is to alter the code writing. To understand the regulation of gene expression in \( T. \) gondii, we and others took advantage of HDAC inhibitors that alter the writing of the histone code and thereby gene transcription (Boyle et al., 2006; Bougdour et al., 2008). We showed that specific inhibition of TgHDAC3 by the cyclopeptide FR235222 disrupts the steady-state level of histone H4 acetylation across the genome inducing derepression of stage-specific genes (Bougdour et al., 2008). In the same study, we isolated parasite lines resistant to the molecule; single-point mutations found in mutagenized parasites target the amino acid T99 in TgHDAC3. We made transgenic \( T. \) gondii carrying these mutations and showed they were sufficient to confer resistance to FR235222. Interestingly, the residue T99 along with the amino acid A98 creates an insertion within the catalytic site of the enzyme that is exclusively conserved in apicomplexan HDAC3 family of proteins and absent in any other eukaryotic HDAC characterized so far. We then provided direct evidence that the HDAC3 family plays a role in gene expression, cell cycle control and consequently in parasite differentiation: drug inhibition of TgHDAC3 prevented the formation of tachyzoite daughter cells while low doses of FR235222 induced conversion in bradyzoites. Indeed, similar to parasites carrying the TgHDAC3T99A and/or TgHDAC3T99I mutations, drug inhibition of TgHDAC3 led to bradyzoite markers expression. FR235222 treatment also blocked the development of synchronized \( P. \) berghei cultures from the ring to the trophozoite stages, and from the trophozoite to the schizont stages, although schizonts were somewhat less sensitive to drug treatment (Bougdour et al., 2009). By this approach, we showed that interfering with the histone code writing helps unravel the role of certain histone PTMs in the control of chromatin activity during \( T. \) gondii stage transition.
The epigenetic language of *T. gondii*

Reading and deciphering the code

A key question is how PTMs patterns are interpreted. By definition, the histone code suggests that PTMs (either single or in combination) are read by effectory molecules that may alter the properties of chromatin by cross-linking nucleosomes (Francis et al., 2004), by enhancing the occupancy of the RNA polymerase preinitiation complex and its associated factors (Vermeulen et al., 2007) or by recruiting further chromatin modification activities (Jenuwein and Allis, 2001). There are two clear major lines of support for this hypothesis in *Apicomplexa*. First, protein domains capable of binding to histone PTMs are well-represented in parasite genomes such as the bromodomain, which functions as an acetyl-lysine binding domain, the chromodomain and the plant homeodomain (PHD) that both bind methyl-lysine. Their distribution is restricted to chromatin-modifying enzymes (HAT, lysine methylase and ATP-dependent remodelling enzymes) and several uncharacterized proteins. Recently, two groups that study malaria parasites showed that the heterochromatin protein 1 (PHHP1) binds exclusively to the repressive mark H3K9me3, by means of a conserved chromodomain (Flueck et al., 2009; Pérez-Toledo et al., 2009). PHHP1 is apparently absent at centromeres (Flueck et al., 2009), which is consistent with H3K9me3 not being enriched at pericentromeric chromatin (Lopez-Rubio et al., 2009). In other eukaryotes like *S. pombe* and *D. melanogaster*, HP1 is an important factor in centromere function and a major constituent of pericentromeric heterochromatin (Grewal and Jia, 2007). In *T. gondii*, while both H3K9me2 and H3K9me3 marks are apparently enriched at the centromere (C.F. Brooks, M. Gissot, K. Kim and B. Striepen, unpubl. work; see H3K9me2 genome-wide data at http://www.toxodb.org), localization of TgHP1 is still missing to formally conclude that they both play a role in centromeric regulation. Altogether, these data could imply important differences in centromere biology between *P. falciparum* and other organisms, including *T. gondii*.

Beyond the centromere biology, the importance of H3K9me3–PHHP1 interaction in *P. falciparum* virulence and host–parasite interactions was pointed out by several studies. Thus, PHHP1-bound genes code not only var genes, but also the majority of *P. falciparum* lineage-specific gene families coding for exported proteins involved in host cell remodelling, immune evasion and cytoadherence (Flueck et al., 2009) in strong correlation with H3K9me3 mapping (Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009).

The second major support for the code hypothesis is the observation that specific modifications take place in predictable and reproducible patterns, that the coexistence of marks within a given histone tail, and within a given chromatin domain may serve in combination to dictate functional outcomes. In some cases, one mark can be sufficient to elicit a specific biologic output; in other cases, multiple marks are required. In *T. gondii*, H3R17me2 was concurrent with acetylation of H3K18, suggesting a possible dual signature for gene activation (Saksouk et al., 2005). In this model, the arginine methyltransferase TgCARM1 appears to work in concert with the acetylase TgGCN5-A, which exhibits an unusual bias for H3K18. Cross-talk between these histone modifications was previously reported in human cells; the HAT CBP/p300 acetylates H3K18 to facilitate CARM1 recruitment and subsequent H3R17 dimethylation (Daujat et al., 2002). Another way to identify new cross-talking marks can be reached by using mass spectrometry data. As described in *P. falciparum* some PTMs coexist on the same peptide (Trelle et al., 2009) to probably act together to achieve a particular biological outcome. In yeast, sumoylation of the histone subunits H2A, H2B and H4 is another negative regulator that shows dynamic interplay with positive-acting histone modifications; histone acetylation and sumoylation can block each other by competition (Nathan et al., 2006). Histone H4 was shown to be sumoylated in both *T. gondii* (Braun et al., 2009) and *P. falciparum* (Issar et al., 2008). Multiple sites were found sumoylated by mass spectrometry in parasite H2Az and H2A outnumbering those present in yeast counterparts (Fig. 1) (Issar et al., 2008). Following the yeast model, the redundancy or multiplicity of sumoylation sites in parasite histones suggest that SUMO is attached to a large number of histone lysines and exerts its regulatory effect (either steric blocking or recruitment of other factors, such as silencing proteins or HDACs) in a manner that is independent of the modified residue (Nathan et al., 2006).

Finally, chromatin modifications are usually termed epigenetic marks; however, it remains to be determined how epigenetic persistence of chromatin states is achieved, and which modifications are heritable. We noticed that parasites engaged in cytokinesis exhibited significant levels of H4K20me1 and H3S10ph, indicating that these markers are probably epigenetically transmitted to the daughter cell in *T. gondii* (Sautel et al., 2007). Remarkably, tachyzoites expressing a mutated version of TgSet8 (F1808Y) that abolishes the monomethylation of H4K20 are not able to progress through the cell cycle, thus indicating that H4K20me1 is required for parasite division (M.A.H., unpubl. data). It has also been observed that environmental conditions, such as passage history or culture conditions, induce phenotypic variations (virulence, initial viability, growth rate, and ability to differentiate) of *T. gondii* strains (Nischik et al., 2001; Khan et al., 2009). As previously thought, it would be of interest to examine whether epigenetic differences exist when comparing the heritable histone PTM patterns of parasites.
freshly harvested from animal with parasites maintained over long-term growth in vitro (Khan et al., 2009). Possibly, the epigenetic status could be altered over passages in vitro and our prediction is that these phenotypic variations should be reversible given that they are not due to DNA sequence differences.

Other factors that influence epigenomes

Crucial questions that need to be addressed are: how chromatin-remodelling enzymes are recruited to target promoters? What features of chromatin influence the ability of TFs to stably interact with the genome, and where do TFs bind to the genome?

First, specific histone modifications could enhance TF recruitment to certain genomic regions. For example, the basal TF TFIID directly binds to the H3K4me3 mark via the PHD of TAF3, and acetylation of H3K9 and H3K14 potentiates this interaction (Vermeulen et al., 2007). This original example reveals a cross-talk between the basal transcription apparatus and transcriptionally active chromatin; thus expanding our view of how histone modifications support transcriptional activation by TFIID.

As a second thought, we suspect that it is the collusion of chromatin marks and sequence-specific DNA-binding factors that all contribute significantly to recruitment and stabilization of other regulatory or effector protein complexes to the chromatin. TFs and their cognate cis-acting binding sites have been difficult to identify in the phylum. Re-analysis of apicomplexan genomes led to the discovery of putative plant-like family of TFs with AP2-like DNA-binding domains (Balaji et al., 2005; Iyer et al., 2008). These intriguing proteins named ApiAP2 have recently become the objects of intense scrutiny by investigators in the field. Twenty-six and 45 AP2-domain containing proteins were predicted in the Plasmodium genus and T. gondii respectively. DNA-binding specificities of two members of P. falciparum were reported by De Silva et al. (2008), and a recent study showed that the P. berghei ApiAP2 (AP2-O) activates gene expression in ookinetes, by binding to specific six-base sequences on the proximal promoter of genes reported to be required for midgut invasion (Yuda et al., 2009). Although it is very likely that these AP2-like domain containing proteins are specific TFs acting in Apicomplexa organisms, no clear and direct demonstration of their role in regulating RNA polymerase activity has been done so far. High-throughput yeast two-hybrid assays done with P. falciparum proteins indicated that ApiAP2 are able to homo- and heterodimerize in various combinations, increasing drastically the absolute number of putative TFs beyond the primary number of ApiAP2 encoding genes (Fig. 2B; Lindner et al., 2009). Dimerization is expected to create diversity in recognition site specificity or influences the regulation of gene expression. ApiAP2 are also embedded in a complex network in which they interact directly with histone acetylase (PF08-0034), demethylase (MAL8P1.111) and PHD-containing protein (PF14-0315) (Fig. 2B). In T. gondii, the AP2-containing protein TgCRC350 (59.m03649) co-purifies with the deacetylase TgHDAC3 (Saksouk et al., 2005). Therefore, it seems very likely that the ApiAP2 proteins are regulators involved in the targeting of histone modifying enzymes in a sequence specific manner.

Studies performed in human cells have shown that non-coding RNAs (ncRNAs) are involved in demarcating active and silent chromatin domains (Rinn et al., 2007). In S. pombe, pericentromeric heterochromatin is strictly dependent on physical interactions with small interfering RNA (siRNA) and the RNA-induced transcriptional silencing machinery (Grewal and Elgin, 2007). We recently discovered in T. gondii that many regions of the 65 Mb genome considered so far as inactive or featureless correspond to sites of considerable small RNA activity. Our data indicate that the small RNA repertoire of T. gondii is exceptionally diverse compared with other unicellular eukaryotic organisms studied so far (M.A.H., unpubl. data). We noticed that DNA loci that generate siRNAs are embedded in heterochromatin domains marked with H4K20 methylation, including centromeric regions. Although a direct demonstration of the role of these siRNAs in the establishment of heterochromatin in T. gondii is still lacking, a noteworthy result was that the T. gondii Argonaute loaded with siRNA co-purified with a TgHDAC3-containing repressor complex (M.A.H., unpubl. data). Together, these observations indicate that small RNAs, in association with histone modifiers, should play a role in heterochromatin formation and most likely in centromere biology in T. gondii. This strikingly contrasts with heterochromatin formation mechanisms in P. falciparum; although a recent report showed that relatively long non-coding RNAs (75 and 175 nucleotides) associate with centromeric regions in P. falciparum, suggesting a role of ncRNAs in chromatin regulation (Li et al., 2007). The mechanisms involved are expected to be very different from those observed in T. gondii given that the RNA interference machinery is not found in the Plasmodium species (Baum et al., 2009).

Conclusion

Epigenetic information contained in chromatin is a fascinating feature used by cells to modulate and extend the genetic code. It should not come as a surprise that obligate intracellular eukaryotic parasites have learnt, through evolution with their host cells, how to subtly use PTMs throughout their life cycle to regulate gene expression. In spite of the similarities of histone modifiers and AP2 TFs, there are substantial mechanistic differences.
between *T. gondii* and *P. falciparum*. *P. falciparum* epigenome is unique in some aspects, stage-specific and highly dynamic as exemplified by the striking differences in the marking of genes in the ring and schizont stages. Such differences might reflect dramatic reorganization of the chromatin and the expected differences in gene expression during parasite differentiation. Mechanistically, the differences in epigenome marking could be explained by a regulation of the availability of certain components of the epigenetic machinery, such as DNA-targeting factor(s) at different stages of the parasite cycle.

*Toxoplasma gondii* has a more extensive chromatin modifying machinery encoded by the genome than *Plasmodium* species and shows striking differences in centromeric marking, not to mention the presence of a sophisticated RNAi machinery. In this regard, siRNAs in *T. gondii* and, likely long RNAs in *Plasmodium*, would certainly add one more layer of complexity to gene regulation models. The emerging picture is that epigenetic changes are linked to stage conversion and virulence, although more data are needed to fully grasp the versatility and the complexity of the mechanism involved. In a not-so-distant future, this field of investigation has the potential to provide crucial new insights into how these eukaryotic parasites of major human relevance regulate the expression of their genome and to reveal new drug targets against these parasites.

**Acknowledgements**

We are grateful to Isabelle Tardieux and Bill Sullivan for critical reading of the manuscript. Hakimi Mohamed-Ali is supported by grants from Centre National de la Recherche Scientifique (ATIP+), the Agence National de la Recherche (ANR; MIME program), Lyon-Biopôle and the Institut National de la Santé et de la Recherche Médicale (Contrat d’Interface).

**References**

Balaji, S., Babu, M.M., Iyer, L.M., and Aravind, L. (2005) Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. *Nucleic Acids Res* **33**: 3994–4006.  
Baum, J., Papenfuss, A.T., Mair, G.R., Janse, C.J., Vlachou, D., Waters, A.P., *et al.* (2009) Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. *Nucleic Acids Res* **37**: 3788–3798.  
Behrke, M.S., Radke, J.B., Smith, A.T., Sullivan, W.J., Jr, and White, M.W. (2008) The transcription of bradyzoite genes in *Toxoplasma gondii* is controlled by autonomous promoter elements. *Mol Microbiol* **68**: 1502–1518.  
Bhatti, M.M., Livingston, M., Mullahpudi, N., and Sullivan, W.J., Jr (2006) Pair of unusual GCN5 histone acetyltransferases and ADA2 homologues in the protozoan parasite *Toxoplasma gondii*. *Eukaryot Cell* **5**: 62–76.  
Blader, I.J., and Saeij, J.P. (2009) Communication between *Toxoplasma gondii* and its host: impact on parasite growth, development, immune evasion, and virulence. *APMIS* **117**: 458–476.  
Bougdour, A., Sautel, C.F., Cannella, D., Braun, L., and Hakimi, M.A. (2008) *Toxoplasma gondii* gene expression is under the control of regulatory pathways acting through chromatin structure. *Parasite* **15**: 206–210.  
Bougdour, A., Maubon, D., Baldacci, P., Ortel, P., Bastien, O., Bouillon, A., *et al.* (2009) Drug inhibition of HDAC3 and epigenetic control of differentiation in Apicomplexa parasites. *J Exp Med* **206**: 953–966.  
Boyle, J.P., Saeij, J.P., Cleary, M.D., and Boothroyd, J.C. (2006) Analysis of gene expression during development: lessons from the Apicomplexa. *Microbes Infect* **8**: 1623–1630.  
Bozdech, Z., Linás, M., Pulliam, B.L., Wong, E.D., Zhu, J., and DeRisi, J.L. (2003) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol* **1**: E5.  
Braun, L., Cannella, D., Pinheiro, A.M., Kieffer, S., Belrhali, H., Garin, J., *et al.* (2009) The small ubiquitin-like modifier (SUMO)-conjugating system of *Toxoplasma gondii*. *Int J Parasitol* **39**: 81–90.  
Chang, B., Chen, Y., Zhao, Y., and Bruck, R.K. (2007) JMJD6 is a histone arginine demethylase. *Science* **318**: 444–447.  
Cui, L., Fan, Q., Cui, L., and Miao, J. (2008) Histone lysine methyltransferases and demethylases in *Plasmodium falciparum*. *Int J Parasitol* **38**: 1083–1097.  
Daujat, S., Bauer, U.M., Shah, V., Turner, B., Berger, S., and Kouzarides, T. (2002) Crosstalk between CARM1 methylation and CBP acetylation on histone H3. *Curr Biol* **12**: 2090–2097.  
De Silva, E.K., Gehrke, A.R., Olszewski, K., León, I., Chahal, J.S., Bulyk, M.L., *et al.* (2008) Specific DNA-binding by apicomplexan AP2 transcription factors. *Proc Natl Acad Sci USA* **105**: 8939–8938.  
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.  
Flueck, C., Bartfai, R., Volz, J., Niederwieser, I., Salcedo-Amaya, A.M., Alako, B.T., *et al.* (2009) *Plasmodium falciparum* heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. *PLoS Pathog* **5**: e1000569.  
Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004) Chromatin matin by a polycomb group protein complex. *Science* **306**: 1574–1577.  
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.  
Garcia, B.A., Hake, S.B., Diaz, R.L., Kauer, M., Morris, S.A., Recht, J., *et al.* (2007) Organismal differences in post-translational modifications in histones H3 and H4. *J Biol Chem* **282**: 7641–7655.  
Gissot, M., Kelly, K.A., Ajluka, J.W., Greaily, J.M., and Kim, K. (2007) Epigenomic modifications predict active promoters...
and gene structure in *Toxoplasma gondii*. PLoS Pathog *3*: e77.

Gissot, M., Choi, S.W., Thompson, R.F., Greally, J.M., and Kim, K. (2008) *Toxoplasma gondii* and Cryptosporidium parvum lack detectable DNA cytosine methylation. *Eukaryot Cell* *7*: 537–540.

Gissot, M., Kim, K., Schaap, D., and Ajioka, J.W. (2009) New eukaryotic systematics: a phylogenetic perspective of developmental gene expression in the Apicomplexa. *Int J Parasitol* *39*: 145–151.

Goldberg, A.D., Allis, C.D., and Bernstein, E. (2007) Epigenetics: a landscape takes shape. *Cell* *128*: 635–638.

Grewal, S.I., and Elgin, S.C. (2007) Transcription and RNA interference in the formation of heterochromatin. *Nature* *447*: 399–406.

Grewal, S.I., and Jia, S. (2007) Heterochromatin revisited. *Nat Rev Genet* *8*: 35–46.

Guenter, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Grewal, S.I., and Jia, S. (2007) Heterochromatin revisited. *Nat Rev Genet* *8*: 35–46.

Iyer, L.M., Anantharaman, V., Wolf, M.Y., and Aravind, L. (2003) Comparative genomics of transcription factors and noncoding RNAs. *Nat Rev Genet* *4*: 26.

Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998) SET domain proteins modulate chromatin domains in eu- and heterochromatin. *Cell Mol Life Sci* *54*: 80–93.

Khan, A., Behnke, M.S., Dunay, I.R., White, M.W., and Sibley, L.D. (2009) Phenotypic and gene expression changes among clonal type I strains of *Toxoplasma gondii*. *Eukaryot Cell* *8*: 1828–1836.

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

Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., et al. (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* *301*: 1503–1508.

Li, B., Carey, M., and Workman, J.L. (2007) The role of chromatin during transcription. *Cell* *128*: 707–719.

Lindner, S.E., De Silva, E.K., Keck, J.L., and Llinás, M. (2009) Structural determinants of DNA binding by a *P. falciparum* ApiAP2 transcriptional regulator. *J Mol Biol* *395*: 558–567.

Lopez-Rubio, J.J., Mancio-Silva, L., and Scherf, A. (2009) Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe* *5*: 179–190.

Miao, J., Fan, Q., Cui, L., Li, J., Li, J., and Cui, L. (2006) The malaria parasite *Plasmodium falciparum* histones: organization, expression, and acetylation. *Gene* *369*: 53–65.

Nathan, D., Ingvarsdottr, K., Sterner, D.E., Bylebyl, G.R., Dokmanovic, M., Dorsey, J.A., et al. (2006) Histone sumoylation is a negative regulator in Saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications. *Genes Dev* *20*: 966–976.

Nischik, N., Schade, B., Dytterska, K., Dlugosz, H., Reichmann, G., and Fischer, H.G. (2001) Attenuation of mouse-virulent *Toxoplasma gondii* parasites is associated with a decrease in interleukin-12-inducing tachyzoite activity and reduced expression of actin, catalase and excretory proteins. *Microbes Infect* *3*: 689–699.

Pérez-Toledo, K., Rojas-Meza, A.P., Mancio-Silva, L., Hernández-Cuevas, N.A., Delgadillo, D.M., Vargas, M., et al. (2009) *Plasmodium falciparum* heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. *Nucleic Acids Res* *37*: 2596–2606.

Prigent, C., and Dimitrov, S. (2003) Phosphorylation of serine 10 in histone H3, what for? *J Cell Sci* *116*: 3677–3685.

Radke, J.R., Behnke, M.S., Mackey, A.J., Radke, J.B., Roos, D.S., and White, M.W. (2005) The transcriptome of *Toxoplasma gondii*. *BMC Biol* *3*: 26.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., et al. (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* *129*: 1311–1323.

Roh, T.Y., Cuddapah, S., Cui, K., and Zhao, K. (2006) The genomic landscape of histone modifications in human T cells. *Proc Natl Acad Sci USA* *103*: 15782–15787.

Roth, S.Y., and Allis, C.D. (1992) Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends Biochem Sci* *17*: 93–98.

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*. *Mol Cell Biol* *25*: 10301–10314.

Salcedo-Amaya, A.M., van Driel, M.A., Alako, B.T., Trelle, M.B., van den Elzen, A.M., Cohen, A.M., et al. (2009) Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of *Plasmodium falciparum*. *Proc Natl Acad Sci USA* *106*: 9655–9660.

Sautel, C.F., Cannella, D., Bastien, O., Kieffer, S., Aldebert, D., Garin, J., et al. (2007) SET8-mediated methylations of histone H4 lysine 20 mark silent heterochromatic domains in apicomplexan genomes. *Mol Cell Biol* *27*: 5711–5724.

Sautel, C.F., Ortet, P., Saksouk, N., Kieffer, S., Garin, J., Bastien, O., et al. (2009) The histone methylase KMTox interacts with the redox-sensor peroxiredoxin-1 and targets genes involved in *Toxoplasma gondii* antioxidant defences. *Mol Microbiol* *71*: 212–226.

Schones, D.E., and Zhao, K. (2008)
approaches to studying chromatin modifications. **Nat Rev Genet** 9: 179–191.

Schreiber, S.L., and Bernstein, B.E. (2002) Signaling network model of chromatin. **Cell** 111: 771–778.

Shogren-Knaak, M., Ishii, H., Sun, J.M., Pazin, M.J., Davie, J.R., and Peterson, C.L. (2006) Histone H4-K16 acetylation controls chromatin structure and protein interactions. **Science** 311: 844–847.

Sibley, L.D., and Ajoka, J.W. (2008) Population structure of **Toxoplasma gondii**: clonal expansion driven by infrequent recombination and selective sweeps. **Annu Rev Microbiol** 62: 329–351.

Smith, C.M., Gafken, P.R., Zhang, Z., Gottschling, D.E., Smith, J.B., and Smith, D.L. (2003) Mass spectrometric quantification of acetylation at specific lysines within the amino-terminal tail of histone H4. **Anal Biochem** 316: 23–33.

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

Sullivan, W.J., Jr, and Hakimi, M.A. (2006) Histone mediated gene activation in **Toxoplasma gondii**. **Mol Biochem Parasitol** 148: 109–116.

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: e84.

Trelle, M.B., Salcedo-Amaya, A.M., Cohen, A.M., Stunnenberg, H.G., and Jensen, O.N. (2009) Global histone analysis by mass spectrometry reveals a high content of acetylated lysine residues in the malaria parasite **Plasmodium falciparum**. **J Proteome Res** 8: 3439–3450.

Turner, B.M. (2000) Histone acetylation and an epigenetic code. **Bioessays** 22: 836–845.

Vermeulen, M., Mulder, K.W., Denissov, S., Pijnappel, W.W., van Schaik, F.M., Varier, R.A., **et al.** (2007) Selective anchoring of TFIIID to nucleosomes by trimethylation of histone H3 lysine 4. **Cell** 131: 58–69.

Whitfield, M.L., Sherlock, G., Saldanha, A.J., Murray, J.I., Ball, C.A., Alexander, K.E., **et al.** (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. **Mol Biol Cell** 13: 1977–2000.

Yuda, M., Iwanaga, S., Shigenobu, S., Mair, G.R., Janse, C.J., Waters, A.P., **et al.** (2009) Identification of a transcription factor in the mosquito-invasive stage of malaria parasites. **Mol Microbiol** 71: 1402–1414.

Zhang, K., Williams, K.E., Huang, L., Yau, P., Siino, J.S., Bradbury, E.M., **et al.** (2002) Histone acetylation and deacetylation: identification of acetylation and methylation sites of HeLa histone H4 by mass spectrometry. **Mol Cell Proteomics** 1: 500–508.