Microreview

Histones and histone modifications in protozoan parasites

William J. Sullivan Jr,1* Arunasalam Naguleswaran1 and Sergio O. Angel2
1Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana. 2Laboratorio de Parasitologia Molecular, IIB-Intech, Conicet-UNSAM, Camino de Circunvalacion Laguna Km. 6, C. C 164 (B7130IIWA) Chascomus, Prov. Buenos Aires, Argentina.

Summary
Protozoan parasites are early branching eukaryotes causing significant morbidity and mortality in humans and livestock. Single-celled parasites have evolved complex life cycles, which may involve multiple host organisms, and strategies to evade host immune responses. Consequently, two key aspects of virulence that underlie pathogenesis are parasite differentiation and antigenic variation, both of which require changes in the expressed genome. Complicating these requisite alterations in the parasite transcriptome is chromatin, which serves as a formidable barrier to DNA processes including transcription, repair, replication and recombination. Considerable progress has been made in the study of chromatin dynamics in other eukaryotes, and there is much to be gained in extending these analyses to protozoan parasites. Much of the work completed to date has focused on histone acetylation and methylation in the apicomplexans and trypanosomatids. As we describe in this review, such studies provide a unique vantage point of the evolutionary picture of eukaryotic cell development, and reveal unique phenomena that could be exploited pharmacologically to treat protozoal diseases.

Introduction
Central to eukaryotic cell physiology is the regulation of the expressed genome. The transcriptome is dynamic, providing the cell flexibility to respond to a broad range of stimuli, but this creates a need for gene expression management systems. One major contributor to gene expression management is chromatin, primarily composed of nucleosomes, which serve as spools to wrap and fold DNA in ways that are generally repressive to transcription. Nucleosomes are comprised of core histones (two paired dimers of H2A/H2B and H3/H4) and linker histone H1, which facilitates the formation of higher-order chromatin structures. The core histones form an octamer structure with the globular C-terminal domains constituting the bulk of the spool (Luger et al., 1997). The N-terminal domains of these histones extend outward and can be post-translationally modified in numerous ways on multiple residues. A battery of proteins exist harbouring domains that can recognize these modified residues, implying that the N-termini of histones may operate as malleable docking sites. The quantity of information that can potentially be encoded on a single nucleosome, or independent nucleosomes acting in concert, is enormous. The idea that distinct downstream events can be directed by histone tail modifications delivered by chromatin remodelling complexes has been coined ‘the histone code’ (Strahl and Allis, 2000). In addition to the expanding list of enzymes that can chemically modify histone tails in this fashion, there is a sizeable group of ATP-dependent remodelers that can relocate nucleosomes or replace their histones with variants (Peterson and Laniel, 2004; Mohrmann and Verrijzer, 2005).

One of the seminal events initiating a revival of the chromatin field arose from experiments conducted with the free-living ciliated protist Tetrahymena thermophila. Brownell et al. purified a histone acetyltransferase (HAT) that possessed homology to a previously identified transcriptional coactivator in yeast called GCN5 (general control non-repressed) (Brownell and Allis, 1995; Brownell et al., 1996). The discovery not only linked a histone modifying protein to transcriptional modulation, but also implied these gene regulatory mechanisms have an ancient origin and are likely to be operational in other protozoa.

The focus of this review is to summarize what is known to date regarding histones and histone modifying
machinery in parasitic protozoa. Alterations in the expressed genomes of pathogenic eukaryotes are critical to virulence and completion of the life cycle. We will highlight studies that demonstrate the relevance of histone modifications with respect to pathogenesis, and discuss how parasite epigenetic mechanisms may be exploited in therapeutic treatments. Most of the work on this subject has been performed in clinically relevant apicomplexan and trypanosomatid parasites. Apicomplexans include *Plasmodium* ssp., the causative agent of malaria, *Cryptosporidium* ssp., a water-borne pathogen that causes diarrhoeal disease, and *Toxoplasma gondii*, an opportunistic infection. Trypanosomatids include *Trypanosoma brucei* and *T. cruzi*, which cause African Sleeping sickness and Chagas’ disease, respectively, and *Leishmania* ssp. (leishmaniasis). Other protozoa referenced include anaerobic pathogens *Giardia lamblia* (giardiasis), *Entamoeba histolytica* (dysentery) and *Trichomonas vaginalis* (trichomoniasis).

**Histone proteins**

Long thought to be uninteresting support proteins for DNA, histones are now experiencing an overdue 15 min of fame now that their regulatory role in gene expression has been realized. Genes encoding the major (canonical) histones are typically present as multiple copies and expressed during S phase. The canonical core histones are some of the most well conserved proteins among eukaryotic species. However, ‘variants’ have been identified for nearly all of the histone proteins, some of which exhibit significant differences in primary sequence compared with their respective canonical version. In addition, histone variants are usually present as a single copy gene and their expression is replication-independent. This raises the possibility that histone variants have different functions in chromatin metabolism. As detailed below, the study of histones and their variants in protozoa has revealed a number of unexpected surprises that not only have intriguing evolutionary implications, but also the potential to lead to vaccine development.

**Histone H1**

Linker histone H1 serves a primary role in chromatin packaging and condensation. Most H1 histones (190–220 amino acids) consist of a central globular domain containing a winged helix motif. This domain is flanked by N- and C-termini rich in proline, lysine, serine and alanine residues that vary dramatically in their length and composition among species, making H1 the most divergent histone. H1 proteins in the protists *Tetrahymena*, *E. histolytica* and the trypanosomatids, are little more than a short lysine-rich protein due to their lacking the central globular domain (Hayashi et al., 1987; Burri et al., 1993; Toro et al., 1993). These H1s are compositionally similar to H1-like proteins in eubacteria or the C-terminal domain of plant and animal H1 (Wu et al., 1986; Kasinsky et al., 2001). Despite the missing winged helix motif, these abbreviated H1s appear functionally equivalent. *Trypanosoma cruzi* H1 can bind linker DNA and induce condensation of nucleosome filaments (Schlimme et al., 1995). No unambiguous H1 has been reported for Apicomplexa, and our bioinformatics searches of available genomes have not been fruitful. The apicomplexan orthologue may simply be too divergent to detect, but it should be noted that *Tetrahymena* deficient for H1 grow normally (Shen et al., 1995). In other words, H1 may not be necessary for certain protozoa.

The state of H1 phosphorylation is associated with different parasite life cycle stages. H1 isolated from proliferating forms (epimastigotes) of *T. cruzi* is not phosphorylated; in contrast, H1 in differentiated/infective forms (trypomastigotes) is phosphorylated and consequently more weakly associated with chromatin (Marques Porto et al., 2002). Interestingly, with respect to virulence, H1 may prove helpful in the design of novel vaccine candidates. Transgenic parasites over-expressing *Leishmania* H1 suffer delays in the cell cycle and differentiation, and are significantly less infective in vivo (Papageorgiou and Soteriadou, 2002; Smirils et al., 2006).

**Histones H2A and H2B**

Among the core histones, H2A has the largest number of variants, and the variants differ among species. Variants H2AZ and H2AX are virtually conserved among eukaryotes, but H2A-Bbd and macroH2A are only seen in vertebrates (Malik and Henikoff, 2003; Kamakaka and Biggins, 2005). In yeast, the major H2A more closely resembles mammalian H2AX than canonical H2A (Malik and Henikoff, 2003). *Drosophila* possesses a single variant (H2AvD) resembling a hybrid of H2AZ and H2AX (van Daal et al., 1988).

DNA damage is associated with monoubiquitylation of H2A and phosphorylation of H2AX (Downs et al., 2000). H2AX has a unique C-terminal motif, SQ(E/D)/Φ, where Φ denotes a hydrophobic residue and Σ is the serine targeted for phosphorylation in response to double-stranded DNA breaks (Rogakou et al., 1998). Figure 1A reflects the diversity of protozoan H2As. As in yeast, *Giardia lamblia*, *E. histolytica* and *T. vaginalis* seem to have replaced canonical H2A with H2AX. Distribution of H2A/H2AX among the apicomplexans is puzzling. *Cryptosporidium* spp. is similar to the aforementioned protists and has replaced H2A with H2AX. In contrast, *Toxoplasma* harbours both canonical H2A and H2AX. Other apicomplexans, including *Plasmodium* ssp., are devoid of H2AX, as reported previously for trypanosomatids (Fig. 1A) (Alsford et al., 2004).
A. Neighbour joining (NJ) trees of H2A, H2B, H3 and H4 sequences were constructed with phylogeny program PAUP* (Phylogenetic Analysis Using Parsimony) version 4b-10 (Sinauer Associates, MA) based on a pair-wise comparison of two sequences according to Kimura’s two parameter model (Kimura, 1980). Ch, Cryptosporidium hominis; Cp, Cryptosporidium parvum; Eh, Entamoeba histolytica; Et, Eimeria tenella; Gi, Giardia lamblia; Li, Leishmania infantum; Lm, Leishmania major; Pb, Plasmodium berghei; Pf, Plasmodium falciparum; Py, Plasmodium yoelii; Ta, Theileria annulata; Tb, Trypanosoma brucei; Tc, Trypanosoma cruzi; Tv, Trichomonas vaginalis; Tg, Toxoplasma gondii; Tp, Theileria parva; Ti, Tetrahymena thermophila. An asterisk denotes that sequence data were obtained from EST and genome project databases using BLASTP and/or BLASTN at http://www.ncbi.nlm.nih.gov, http://www.toxodb.org, http://www.plasmodb.org, http://tigrblast.tigr.org/tgi. Accession numbers are available as supplementary data.

B. Comparison of the C-terminal amino acids (final 10–12 residues) of various Apicomplexa H2A homologues. Underscored residues refer to the SQ(E/D) consensus motif of H2AX.
Histones H3 and H4

There is probably no better example of evolutionary inertia than that of H3 and H4, perhaps due in part to the extensive post-translational modifications that can occur on these histones to affect gene expression. Yet again, some of the elder eukaryotes continue to present intriguing surprises. Most organisms have three H3 class histones: (i) canonical H3, (ii) the very similar variant H3.3, associated with transcriptionally active loci and (iii) CenH3, which incorporates into the centromere to facilitate kinetochore formation. Apicomplexans express both canonical H3 and H3.3 (Sullivan, 2003) (Fig. 1A), and a recent study suggests CenH3 to be present in *Plasmodium falciparum* (Miao et al., 2006). CenH3 homologues for other apicomplexans have not been reported, but gene prediction cg04-2030 represents a candidate for *Cryptosporidium parvum*. A third H3-like prediction (42. m00059) is in the *Toxoplasma* database, but studies are required to test if this is CenH3. Trypanosomatids also have at least two H3 histones, the canonical and an atypical H3 variant (H3v) that does not clearly resemble H3.3 or CenH3 (Fig. 1A) (Alsford and Horn, 2004). Moreover, *T. brucei* H3v is present in telomeric DNA and is not essential for parasite viability (Lowell and Cross, 2004).

Consistent with other eukaryotes, protozoa possess two canonical H4, but no variant. In Apicomplexa H4 is unremarkable; however, H4 sequences of other protozoa are more divergent than anticipated for such an evolutionarily rigid protein (Fig. 1A). The trypanosomatids exhibit the largest degree of H4 divergence, some even harbouring a novel 'orphan' variant H4 (Alsford and Horn, 2004). *Entamoeba* and *Giardia* H4 contain more than the usual four lysines typically targeted for acetylation, and recent studies on the former suggest that all seven lysines are acetylated (D. Eichinger, pers. comm.). Whether these curious differences are trivial or have relevant functional consequences remains to be investigated.

In short, the histone complement in protozoan parasites displays important differences in its overall composition and in the primary sequences of certain individual histones, which could produce new post-translational modification or ‘marks’. Examples of novel histone modifications reported for *T. brucei* include N-methylation of the N-terminal alanine in H2A, H2B and H4, as well as hyperacylation of the C-terminus of H2A (Janzen et al., 2006a).

**ATP-dependent chromatin remodelling**

The replacement of canonical histones with variants or the relocation of nucleosomes can dramatically alter the topology of chromatin. Such restructuring can be mediated by complexes containing SWI2/SNF2 ATPases, a large family of proteins classified into four groups based on the presence of additional domains: Sfn2 (bromo-domain), ISWI (SANT domain), Mi-2 (chromodomain) and SWR1/SRCAP (disrupted ATPase domain) (Mohrmann and Verrijzer, 2005). SWI2/SNF2 factors have been documented in Apicomplexa, specifically an ISWI homologue in *P. falciparum* and SRCAP homologues in *Toxoplasma* (TgSRCAP), *C. parvum* and *P. falciparum* (Ji and Arnot, 1997; Sullivan et al., 2003). Bioinformatics survey of the *Toxoplasma* database reveals at least 15 other presumed SWI2/SNF2s, including two putative Sfn2s (but lack bromodomains), two ISWI (one with SANT domain, the other...
with AT hook) and one Mi-2 with chromodomain (Sullivan and Hakimi, 2006). Other apicomplexans have an appreciable number of putative SWI2/SNF2 factors as well; *C. parvum* and *P. falciparum* have 14 and 11 SWI2/SNF2 ATPases respectively (Templeton et al., 2004).

There is evidence in *T. brucei* that a SWI2/SNF2-like protein is important for virulence mechanisms by virtue of contributing to the formation of the modified thymine base (called J). The J base may be involved with epigenetic repression of variant surface glycoprotein (VSG) expression, thus contributing to antigenic variation. This SWI2/SNF2 was named J binding protein 2 (JBP2) and is proposed to interact with chromatin in such a way that allows J-biosynthetic enzymes better access (DiPaolo et al., 2005).

Like its human counterpart, TgSRCAP enhances CREB-mediated expression in the presence of CREB-binding protein (CBP) when cotransfected into HeLa cells (Sullivan et al., 2003). However, as it is now evident that Apicomplexa have no CBP or CREB, the role of TgSRCAP remains unclear. TgSRCAP may be relevant in stage differentiation as implied by reverse transcription polymerase chain reaction analysis showing that its mRNA levels increase during *in vitro* differentiation (Sullivan et al., 2003). The yeast SWR1 (related to SRCAP) complex can replace H2A with H2AZ in nucleosomes (Mizuguchi et al., 2004), and it will be of interest to determine if TgSRCAP can do the same.

**Histone marks and modifying enzymes**

**The histone code**

The abundance of post-transcriptional modifications on histones has given rise to the histone code hypothesis, which specifies that these modifications (or marks) constitute a cellular language that produces specific downstream effects (Strahl and Allis, 2000). Numerous transcriptional regulators have domains that are capable of recognizing specific histone modifications, which is consistent with the existence of a histone code. Examples include bromodomains that interact with acetylated lysines, chromodomains and tudor domains that bind methylated histones, and macro domains that recognize ADP-ribose moieties (de la Cruz et al., 2005). Histone marks and modifying enzymes in other eukaryotes have been extensively reviewed (e.g. Peterson and Laniel, 2004), so we will focus on the chromatin modifiers in parasitic protozoa in the context of parasite virulence and differentiation.

**Apicomplexans**

A considerable number of histone modifications have been documented in *P. falciparum* and *Toxoplasma* (Fig. 2). Acetylated histone residues have also been detected in *Cryptosporidium* by immunoblot (D. Rider, pers. comm.) (Fig. 2). *Toxoplasma* possesses at least one putative homologue of every histone modifier that has been identified in other species (Sullivan and Hakimi, 2006). In short, *Toxoplasma* has the necessary equipment and corresponding histone substrates to deliver virtually every histone modification described, implying that this level of gene regulation has a very ancient origin. A noteworthy feature regarding *Toxoplasma* HATs is the presence of two GCN5 family members (TgGCN5-A and -B) that exhibit different H3 acetylation activities and differentially associate with two *Toxoplasma* ADA2 homologues *in vitro* (Bhatti et al., 2006). Pairs of GCN5 HATs (GCN5 and PCAF) and ADA2 coactivators are common in multicellular eukaryotes, but not protozoa or yeast. It has been proposed that TgGCN5-A is a duplication of TgGCN5-B that may have evolved a specialized function. *Toxoplasma* tachyzoites have no discernible phenotype when TgGCN5-A is knocked out, and the loss has no impact on virulence in mice (Bhatti et al., 2006).

A single GCN5 HAT has also been documented in *P. falciparum* (Fan et al., 2004). An unusual feature that apicomplexan GCN5s share is a lengthy N-terminal extension that has no homology to known proteins, including one another. Functions of the N-terminal extension have been elucidated for *Toxoplasma* GCN5-A, and include nuclear localization and protein–protein interactions (Bhatti and Sullivan, 2005). Apicomplexa also possess MYST family HATs, which have a substrate preference for H4 (Smith et al., 2005). Histone deacetylases (HDAC), which remove acetyl groups from lysine residues and restore chromatin to its repressive state, have also been reported (Joshi et al., 1999; Duraisingh et al., 2005; Freitas-Junior et al., 2005; Saksouk et al., 2005).

Relevant to pathogenesis, epigenetics was proposed to be a mechanism behind the allelic exclusion that results in the expression of a single variant surface antigen (var) gene in *P. falciparum* (Scherf et al., 1998; Deitsch et al., 1999; Voss et al., 2000). Recent studies have validated that histone modifications contribute significantly to antigenic variation. Specifically, activation of a specific telomere-associated var gene correlates with the removal of the HDAC Sir2 and subsequent acetylation of histones (Duraisingh et al., 2005; Freitas-Junior et al., 2005). Histone acetylation state also correlates with *Toxoplasma* tachyzoite to bradyzoite differentiation. Chromatin immunoprecipitation (ChIP) assays of tachyzoites demonstrated that TgGCN5-A is present at tachyzoite-specific promoters while TgHDAC3 is at bradyzoite-specific promoters, presumably keeping the corresponding genes active or silent respectively (Saksouk et al., 2005). Additionally, microarray analysis of *Toxoplasma* treated with an HDAC inhibitor further support an important role for...
Histone acetylation in life cycle stage conversion (Boyle et al., 2006).

Histone methylation can occur on lysine or arginine residues, and the effect on gene transcription can be positive or negative. Histone methyltransferases (HMTs) that can modify lysines (SET-domain HMTs) and arginines (PRMTs) have been identified in Toxoplasma (Sullivan and Hakimi, 2006), and P. falciparum histones examined by mass spectrometry exhibit methylated residues (Miao et al., 2006). Toxoplasma PRMT1 and PRMT4 (TgCARM1) methylate H4 arginine 3 and H3 arginine 17 respectively (Saksouk et al., 2005) (Fig. 2). TgCARM1 was demonstrated to deliver a mark of gene activation in tachyzoites and bradyzoites. Pretreatment of parasites with an inhibitor of CARM1 prior to infection of host cells increases the frequency of bradyzoite conversion, suggesting a potential connection between HMT activities and differentiation (Saksouk et al., 2005). Additional histone methylation marks detected in Toxoplasma include H3 lysines 4 and 9, and H4 lysine 20 (Sullivan and Hakimi, 2006). Further support for the involvement of histone methylation in differentiation is evident in the observation that tri-methylated H3 lysine 4 is enriched at tachyzoite-specific promoters during that stage of the life cycle, and becomes enhanced at bradyzoite-specific promoters after differentiation is induced (Saksouk et al., 2005).

Trypanosomatids

The investigation of the chromatin marks in the trypanosomatids promises to be very intriguing given the rather dramatic divergence in the histone sequences relative to other eukaryotes (fellow protozoa included), and the unorthodox nature of gene transcription in these parasites (Fig. 2). Protein coding genes in trypanosomatids are organized into polycistronic transcription units that produce polycistronic precursor RNAs, which are subsequently processed to monocistronic mRNAs via transsplicing and polyadenylation (Belli, 2000).

Mass spectrometry analysis of T. brucei core histones reveals a striking absence of many well-conserved post-translational modifications. T. brucei histones display acetylation on multiple lysines of the C-terminus of TbH2A and abundant levels of methylated N-terminal alanines on H2A, H2B and H4 (Janzen et al., 2006a) (Fig. 2). These
modifications are unique in that they have not been reported in other organisms. There is evidence for at least two previously known histone marks: tri-methylation of H3 lysine 4 (Alsford and Horn, 2004) and di/tri-methylation of H3 lysine 76 (79 in other species) (Janzen et al., 2006b).

In T. brucei, the HDACs DAC1 and DAC3 are essential and DAC4 is required for cell cycle progression (Ingram and Horn, 2002). A noteworthy feature of the T. brucei Sir2 (TbSIR2RP1) is that it possesses histone ADP-ribosyltransferase activity in addition to HDAC activity (Garcia-Salcedo et al., 2003). A subclass of poly-ADP-ribose polymerases (PARPs) ribosylate histones in response to DNA damage in mammals, and TbSIR2RP1 has been linked to DNA repair (Garcia-Salcedo et al., 2003). TbSIR2RP1 is also subject to auto-ADP-ribosylation in vitro, and Drosophila Sir2 activity can be modulated by the addition of an ADP-ribose moiety to Sir2 itself (Tulin et al., 2006). ADP-ribosylated Sir2 migrates to a single structure in the perinuclear cytoplasm, creating the potential for Sir2-silenced genes to become activated. It will be of interest to determine if TbSIR2RP1 is auto-ADP-ribosylated in vivo and what consequence this has on localization.

Similar to Plasmodium, T. brucei employs an immune invasion strategy that involves mono-allelic expression of a telomeric VSG gene, which consequently requires reversible repression of other telomere-proximal VSGs (Borst and Ulbert, 2001). Studies have demonstrated a specific role for chromatin remodelling in the developmental downregulation of a VSG expression site (Navarro et al., 1999; Glover and Horn, 2006). Deciphering of the histone code in trypanosomatids will undoubtedly advance our understanding of antigenic variation. Future directions that may hold particular relevance in this case should include a closer look at histone ubiquitination in trypanosomes. An unusually high concentration of a ubiquitinated histone-like protein has been observed in T. cruzi (Reverol et al., 1997). This may be significant as the ubiquitination of yeast H2B has been linked to telomere-associated gene silencing (Sun and Allis, 2002).

DOT1 methyltransferases have recently been implicated in cell cycle regulation in T. brucei. In other species, DOT1 targets H3 lysine 79, a modification associated with transcription activation, meiotic checkpoint control and DNA repair. In T. brucei, DOT1A and DOT1B mediate di- and tri-methylation of the corresponding lysine in TbH3 (K76), respectively, and the methylation status of this residue effects the trypanosome cell cycle and differentiation (Janzen et al., 2006b).

In short, despite the unusual transcription process and histone sequences in the trypanosomatids, histone modifications and modifiers exist. This suggests that a histone code is employed by these parasites, but that it is likely to be a different dialect.

Other parasitic protozoa

Studies in other parasitic protozoa are limited at present, but initial reports indicate that chromatin modification is conserved in these early branching eukaryotes as well. Western blot analysis shows that basic nuclear proteins exist in acetylated forms in E. histolytica, and HAT/HDAC homologues were subsequently identified (Ramakrishnan et al., 2004). E. histolytica possesses at least one GCN5- and one MYST-family HAT, and a class I HDAC. Curiously, butyrate, which normally inhibits HDAC activity and leads to histone hyperacetylation, induces hypoacetylation of H4 in Entamoeba (D. Eichinger, pers. comm.). This may reflect an important adaptation that allows this parasite to survive in the presence of butyric acid in the colon.

Additional evidence suggesting that epigenetics is an important component of gene regulation in E. histolytica comes from studies examining a peculiar silencing effect on the amoebapore A (Ehap-a) gene after transfection with plasmid containing a portion of the 5’ upstream region of Ehap-a and a short interspersed nuclear element (SINE1). Genes similar to Ehap-a also become silenced after transfection, and the silencing remains in effect even if plasmid is removed (Bracha et al., 2006). ChiP analysis suggests transcriptional inactivation via demethylation of H3 lysine 4 at the domain of the Ehap-a gene, implicating a role for epigenetics in this phenomenon (Anbar et al., 2005). It is vital that further research is performed in this area, as transcriptional modulation of virulence genes is believed to be one mechanism for regulating pathogenicity of E. histolytica (Ramakrishnan et al., 2004).

Final thoughts: breaking the code

The modulation of the expressed genome is critical to cellular viability. Parasitic protozoa, in particular, have complex life cycles and must respond rapidly to changes in their environments. Genomes of pathogenic protists generally display a remarkable lack of conventional DNA-binding transcription factors, which are considered the key components to gene expression regulation in most other eukaryotes. It is possible that the functional equivalents of these transcription factors in protozoa are so distal they are simply beyond the reach of bioinformatics. But analysis of the histones and chromatin remodelling machinery presents a second possibility that parasites rely more on epigenetics to communicate cellular information.

Emerging studies are revealing that several different mechanisms exist to organize and convey epigenetic information, which are not likely to be mutually exclusive: (i) nucleosome–DNA interactions that can be modulated by addition/removal of chemical moieties or by the energy of ATP hydrolysis, (ii) histone codes, (iii) replacement of
canonical histones with variants, (iv) chemical modification of DNA itself (methylation or J glucosyl-bases), and (v) subnuclear localization. A summary schematic of these epigenetic mechanisms and their presence in parasitic protozoa is depicted in Fig. 3.

Genesis of the histone code
Study of the histone modifications and modifiers in these early branching eukaryotes will certainly contribute to our understanding of the origins of histone codes, and how this code can be manipulated to help organisms adapt to various environments. Initial observations have already raised a number of intriguing questions. Genomic data suggest an absence of H2AX in trypanosomatids and many Apicomplexa. Assuming this is not an artefact of gene prediction, it will be of interest to examine how these cells mediate DNA repair compared with others that phosphorylate H2AX, and how they mediate DNA recombination and genome stability. Similarly, there is a curious lack of an unambiguous CenH3 in some Apicomplexa and trypanosomatids, despite the presence of centromeres.

Noting the congruities between the histones of early versus later branching eukaryotes highlights crucial features of the histone code. The resistance to evolutionary change exhibited by H4 is particularly strong, perhaps indicating that it plays essential roles in cellular physiology and gene regulation. A more specific example is the absolute conservation of H3 lysine 4 and its ability to be methylated.

Learning the language
The first step in breaking a code is learning the language. As exemplified in Fig. 2, there is much work remaining to elucidate the nature of the histone modifications in protozoan parasites. Apicomplexan H3 and H4 tails exhibit little divergence from other eukaryotes. In contrast, the lysine and arginine residues that are susceptible to modification in other eukaryotes are not unambiguously conserved in Entamoeba, Giardia and the trypanosomatids. However, there is no shortage of lysines and arginines in the histone tails of these protozoa, making it tempting to speculate that they are indeed modified in a similar fashion. For example, H3 lysine 9 in apicomplexans and other eukaryotes is well conserved and subject to both acetylation and methylation. Trypanosomatids have a lysine at position 10 instead, but it remains to be determined if it can be modified as lysine 9 is in other eukaryotes (Fig. 2). Another feature revealed by the alignments is the absolute conservation of H3 lysine 4. Considered with its proclivity for methylation among the early branching eukaryotes, modification of H3 lysine 4 may be suggestive of a universal histone mark of great importance. A dual approach to identify the histone modifications and characterize the predicted chromatin modifiers should be employed to effectively advance the field.

Eating away at the code: fluorescence in situ hybridization (FISH) and ChIPs
The optimization of ChIPs for Plasmodium and Toxoplasma has been a key development in advancing research of chromatin dynamics in these protozoa (Duraisingh et al., 2005; Freitas-Junior et al., 2005; Saksouk et al., 2005). The high conservation of apicomplexan histones has facilitated the analysis of the potential histone code in these organisms. The remarkable diversity of the histone tails in other parasites complicates similar analyses as antibodies are not available.
Another powerful tool to study epigenetic phenomena is FISH. FISH has been instrumental in the study of monoallelic expression of *P. falciparum var* genes and *T. brucei* VSG genes, demonstrating that subnuclear regions impact transcriptional activity (Chaves et al., 1998; Figueiredo et al., 2002; Ralph et al., 2005). FISH protocols have also been developed for *Giardia* and *Entamoeba* (Willhoeft and Tannich, 2000; Yu et al., 2002).

‘Braking’ the code

There is a potential goldmine of novel therapeutic targets involving regulation of gene expression. In other words, stopping the parasite from effectively executing its histone code would result in disinformation and misregulation of gene expression. Two *T. brucei* HDACs and DOT1A are essential, and multiple chromatin remodelers are refractory to allelic disruption in *Toxoplasma* (e.g. TgCARM1, TgMYST-A and TgSRCAP) (W.J. Sullivan, unpubl. obs.; Ingram and Horn, 2002; Saksouk et al., 2005; Smith et al., 2005; Janzen et al., 2006b). Pharmacological support for the importance of histone acetylation in protozoa is evident in the antiparasitic properties of HDAC inhibitors (Darkin-Rattray et al., 1996; Kwon et al., 2003). Studies with apicidin reveal noteworthy differences between the HDACs of various parasites; although effective against apicomplexans, apicidin is inactive against *Giardia lamblia*, *Trichomonas foetus* and trypansomatids. Another class of HDAC inhibitors, aroyl-pyrrolyl-hydroxamides, exhibit both antimalarial and antileishmanial activities (Maï et al., 2004).

Protozoan histones may also be exploitable in novel therapeutic approaches as immunogens or by way of creating attenuated strains for vaccine applications. Transgenic parasites over-expressing H1 have been examined as a vaccine candidate for leishmaniasis (Papageorgiou and Soteriadou, 2002). Further, the prophylactic value of a cocktail consisting of the four core *Leishmania infantum* histones was evaluated in the murine model of cutaneous leishmaniasis. DNA vaccination with these *Leishmania* histone genes results in a specific Th1-like response during *L. major* infection that contributes to the resistance of vaccinated mice (Iborra et al., 2004).

Factors that govern virulence are often intimately associated with changes in gene expression, leading to the hypothesis that alterations in histone proteins contribute to virulence mechanisms. Several lines of evidence have been discussed that are consistent with this hypothesis. It has been established in multiple protozoa that there is an inverse correlation between HAT and HDAC activities to modulate stage-specific genes during differentiation and gene expression pertinent to antigenic variation. Research in the near future is likely to reveal more precise patterns of histone acetylation and discovery of additional histone modifications germane to these processes. In *Toxoplasma*, a CARM1 inhibitor influences bradyzoite differentiation, suggesting a role of histone methylation in the process as well. The formation of the stage specific base J in bloodstream form *T. brucei* appears to be initiated by chromatin remodelling complexes, and DOT1B is essential for developmental transition to procyclic forms (DiPaolo et al., 2005; Janzen et al., 2006b).

In addition to resolving the parasite histone codes, attention needs to be focused on the targeting of the remodelers to gene loci. In higher eukaryotes, this is mediated in part by DNA-binding transcription factors, which recruit multisubunit complexes containing the chromatin modifying enzyme. The paucity of such factors in early branching eukaryotes implies the targeting mechanism may differ. It cannot be dismissed that a small array of transcription factors operate in protozoa, but other methods should be entertained. For example, targeting can be mediated by interaction of non-coding RNAs with chromatin remodelling complexes, and the redistribution of genes to the nuclear periphery has also been shown to be important for expression (Cabal et al., 2006; Sanchez-Elsner et al., 2006).

Continued study of the histones and histone modifications in protozoan parasites has much to offer in aiding our quest to understand the biological role of this phenomenon in late-branching eukaryotes. Evidence is strong that these modifications function as a language to direct cellular responses. If we can learn that language, we will be better equipped to understand the parasites.

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Note added in proof

A relevant study has recently been published describing that antigenic variation in *Giardia lamblia* also involves epigenetic mechanisms [Kulakova, L., Singer, S.M., Conrad, J., and Nash T.E. (2006) Epigenetic mechanisms are involved in the control of *Giardia lamblia* antigenic variation. *Mol Microbiol* 61: 1533–1542.]

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