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Journal

Briefings in Functional Genomics, 18(5)

ISSN

2041-2649

Authors

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Publication Date

2019-09-24

DOI

10.1093/bfgp/elz005

Peer reviewed

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Briefings in Functional Genomics, 18(5), 2019, 302–313

doi: 10.1093/bfgp/elz005
Advance Access Publication Date: 26 April 2019
Review paper

The role of epigenetics and chromatin structure in transcriptional regulation in malaria parasites

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Abstract

Due to the unique selective pressures and extreme changes faced by the human malaria parasite *Plasmodium falciparum* throughout its life cycle, the parasite has evolved distinct features to alter its gene expression patterns. Along with classical gene regulation by transcription factors (TFs), of which only one family, the AP2 TFs, has been described in the parasite genome, a large body of evidence points toward chromatin structure and epigenetic factors mediating the changes in gene expression associated with parasite life cycle stages. These attributes may be critically important for immune evasion, host cell invasion and development of the parasite in its two hosts, the human and the *Anopheles* vector. Thus, the factors involved in the maintenance and regulation of chromatin and epigenetic features represent potential targets for antimalarial drugs. In this review, we discuss the mechanisms in *P. falciparum* that regulate chromatin structure, nucleosome landscape, the 3-dimensional structure of the genome and additional distinctive features created by parasite-specific genes and gene families. We review conserved traits of chromatin in eukaryotes in order to highlight what is unique in the parasite.

Key words: malaria; gene regulation; transcription; chromatin structure; epigenetics

Malaria and *P. falciparum*

According to estimates in recent years, around 200 million cases of malaria infection occur annually, spread over 91 countries and result in more than 400 000 deaths [1]. Ninety percent of malaria cases and deaths occur in sub-Saharan Africa, but the disease is also widespread in Southeast Asia and South America. More than two-thirds of malaria deaths occurred in children under 5.

Multiple apicomplexan parasites of the genus *Plasmodium* can cause malaria in humans. These include *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi* and *P. ovale*, which in truth likely represents two distinct species, *P. o. curtisi* and *P. o. wallikeri* [2]. However, because *P. falciparum* is responsible for the vast majority of human fatalities [1], it has received the bulk of research attention. The unicellular eukaryotic parasite traverses a complex life cycle involving a mosquito vector and a human host, as well as both asexual and sexual replication cycles. Its genome is composed of 23 million base pairs per haploid genome, arranged into 14 chromosomes [3]. The most striking trait of the parasite genome is its high AT-content (prevalence of adenine-thymine as opposed to guanine-cytosine base pairs) of around 80% and rising to 90–95% in intergenic regions, making this the most AT-rich eukaryotic genome yet sequenced. Changes in the parasite's needs and environment occur as the parasite progresses through its life cycle, necessitating large-scale shifts in gene expression [4, 5]. While several AP2 transcription factors (TFs) have been identified as potential master regulators of transcription and stage transitions [6–12], the 27 putative *Plasmodium*-specific TFs remain extremely low in number compared to other eukaryotes to regulate the expression of ∼5500 parasite protein-coding genes [6, 9]. As an example, gene expression in the similarly sized yeast genome has been shown to be regulated by 169 specific TFs [13]. As a result, despite the existence of a few other types of identified DNA-binding factors [14–16], researchers remain perplexed as to how such a small number of TFs can govern a complex gene expression program. A good deal of evidence now points toward additional mechanisms related to epigenetics and

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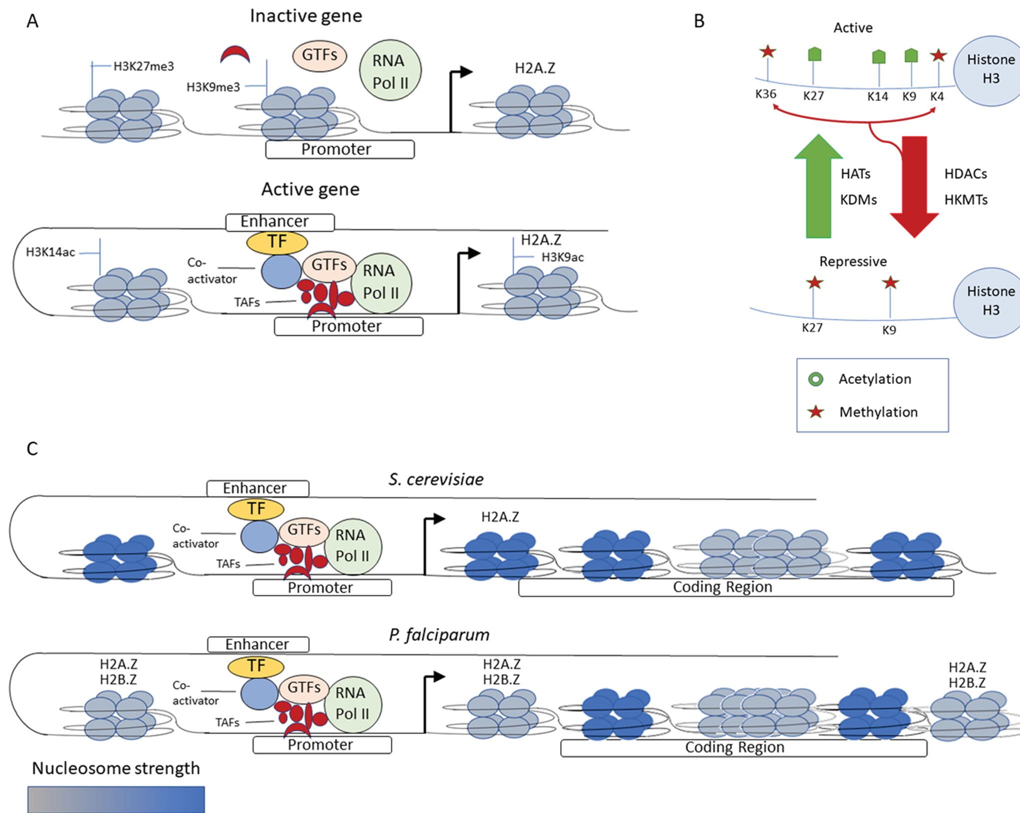

Figure 1. Schematic representation of chromatin structure and transcription in eukaryotes. (A) Nucleosome occupancy at promoter regions modulates level of transcription of eukaryotic genes by altering accessibility of the region for the basal transcription machinery. (B) Histone H3 on nucleosomes in chromatin can be post-translationally modified at specific lysine residues on its N-terminal tail. HATs and lysine demethylases (KDMs) promote acetylation, while histone deacetylases (HDACs) and histone lysine methyltrasferases (HKMTs) promote methylation. (C) Nucleosome landscapes of active genic regions differ between *Saccharomyces cerevisiae* and *P. falciparum*. In yeast, strongly positioned nucleosomes flank the promoter region and tend to include more weakly binding histone variant H2A.Z, which helps to open the region for transcription. In *P. falciparum*, not only H2A.Z but parasite-specific H2B.Z variants are found near genes, which may reflect a method to bind AT-rich DNA. Strongly positioned nucleosomes are found at the beginning and end of the coding region, with more randomly placed nucleosomes throughout the coding region than is typical in eukaryotes.

chromatin structure controlling gene expression in the parasite. This review aims to bring the reader up-to-date on the current state of research in epigenetics and chromatin structure in malaria parasites, how they may affect gene expression through transcriptional regulation and how this line of inquiry could lead to new ways to combat this dangerous parasite.

General transcriptional activity and chromatin structure in eukaryotes

To examine unique features of transcription and chromatin structure in the human malaria parasite, *P. falciparum*, it is useful to understand the typical characteristics of gene regulation and chromatin landscape in eukaryotes. Inside the eukaryotic cell nucleus, DNA is tightly wrapped around groups of histone proteins to form nucleosomes. These nucleosomes are packed tightly into a compact fiber called chromatin. Chromatin generally exists in one of two states: euchromatin, which is relatively open and accessible to transcriptional machinery, or heterochromatin, which is closed and transcriptionally repressive. Post-translational modifications (PTMs) of histones, together with other protein complexes, are known to alter chromatin structure and affect levels of transcription (Figure 1A).

In eukaryotes, transcription of protein-coding genes is catalyzed by RNA polymerase II, a multiprotein complex composed of an enzyme and associated subunits. The complex is recruited by other general TFs such as TFII components, to promoter regions upstream of genes, which leads to the formation of the basal preinitiation complex (PIC) that can activate transcription. However, the tight compaction of nucleosomes creates an obstacle for the PIC to access promoter regions. In organisms such as yeast and human, genome-wide nucleosome mapping experiments have demonstrated the presence of conserved features that increase accessibility of the PIC to these promoter regions. For example, genic regions are typically more nucleosome-dense compared to intergenic regions, and promoters have a distinct nucleosome-free region to allow direct accessibility for general TFs and RNA Polymerase II (Figure 1A) [17, 18]. Additionally, the histone variant H2A.Z is typically seen at active promoters and is strongly positioned at −1 and +1 nucleosomes flanking the transcription start site (TSS) [19–21]. Furthermore, dynamic nucleosome repositioning in the promoter regions of active genes has been associated with changes in gene expression [22]. All together, these results suggest that nucleosome density and precise positioning at promoter and transcription start/stop sites regulate gene expression in eukaryotes by modulating the ability of the basal transcription machinery to access their binding sites.

Apart from these general trends, the eukaryotic cell utilizes at least two other means to precisely regulate nucleosome positioning and thus gene expression. First, a high number of

specific TFs can be activated to bind specific DNA-binding motifs and recruit ATP-dependent chromatin-remodeling enzymes that can actively deposit, remove or relocate nucleosomes to fine-tune expression of genes in a given genomic region [22]. Second, histone proteins that make up nucleosomes can be post-translationally modified, altering chromatin contacts or recruiting other proteins to chromatin. For example, acetylation of certain residues on histone tails such as lysine 9 or 14 on histone 3 (H3K9ac or H3K14ac) can be performed by proteins with histone acetyltransferase (HAT) domains [23, 24]. Although histone acetylation can have multiple functions, it is often associated with increased transcriptional activation by weakening the histone-DNA interaction and making chromatin more accessible to binding [23, 24]. On the other hand, trimethylation of histone H3 at lysine 9 or 27 (H3K9me3 or H3K27me3) has been implicated in transcriptional repression [23, 24]. The action of HKMTs and HDACs leads to this methylated state, while that of KDMs opposes it (Figure 1B) [23–25].

Over the years, it has become clear that in addition to histone PTMs, long noncoding RNAs (lncRNAs) play a role in regulating gene expression. This is a feature shared by all eukaryotes. For example, in mammals, the X-inactive specific lncRNA, Xist (X-inactive specific transcript), acts as a major effector of the X inactivation process in mammals [26–28]. Xist binds in *cis* and becomes incorporated into chromatin structure at its targets [29]. In this case, lncRNA Xist promotes heterochromatin formation by recruitment of a DNA-methyltransferase and histone-modifying enzymes [30–32] that lead to chromosome inactivation.

Finally, chromatin in the nucleus is not arranged in a linear fashion from beginning to end of the genome, but rather takes up 3-dimensional (3D) space in the nucleus. In organisms ranging from metazoans to single-celled eukaryotes like *P. falciparum*, 3D genome structure significantly contributes to regulation of gene expression [33]. For example, the physical interaction of distal regulatory elements with their target promoters is facilitated by formation of chromatin loops that bring these linearly separated regions together. Genes regulated in similar ways may also cluster into separate domains, with active genes separated from inactive genes [34].

Transcriptional activity and chromatin structure in *P. falciparum*

While the most conserved elements of eukaryotic transcriptional machinery are present in *P. falciparum*, parasite-specific features affect how transcription is carried out and regulated. Similar to other eukaryotes, the parasite possesses the RNA polymerase II complex and associated general TFII TFs, including the TATA-binding protein (TBP) that is part of the TFIID subunit [9, 35]. However, while TFIID in most eukaryotes possesses TBP-associated factors (TAFs) with histone fold domains, the relatively few TAFs that have been identified in *P. falciparum* do not contain the histone fold domain [35]. As the histone fold domain is involved in heterodimerization of TAFs, the lack of this domain in parasite TAFs suggests a divergent TFIID complex compared to other eukaryotes. The low number of TAFs may also point to alternative mechanisms being more important for transcriptional regulation in parasite.

At the epigenetic level, chromatin and nucleosome organization in *Plasmodium* show reduced stability as compared to chromatin and nucleosome organization in higher eukaryotes, reflecting an increased accessibility of the parasite genome [36]. One factor that may contribute to the lower stability nucleosomes and overall openness of chromatin structure in *Plasmodium* is the apparent absence of linker histone H1 in Apicomplexa [37, 38]. It remains possible that a homolog divergent from those of other eukaryotes exists, but as no clear chromatin condensation has ever been observed in *Plasmodium* during mitosis [39, 40], it is likely that even a divergent homolog of the histone H1 may not be required in *Plasmodium*. Most importantly, with the exception of the telomere ends and a few internal loci, which are marked by the repressive histone mark H3K9me3 and heterochromatin protein 1 (PfHP1) [41–43], most of the chromatin in the nucleus exists as euchromatin with active histone modification marks such as H3K4me3, H3K9ac and H4K8ac [41, 44, 45] observed through the genome. Only a few parasite-specific gene families including gene families coding for clonally variant antigens, proteins involved in erythrocyte invasion and other key proteins such as the gametocyte-promoting TF, PfAP2-G, are known to be maintained in one or more heterochromatin cluster(s) around the periphery of the parasite nucleus [41, 44, 46–49]. These particular features are addressed in more detail below.

Genome-wide nucleosome and histone trends in *P. falciparum*

Nucleosome mapping and other techniques have shown that *P. falciparum* retains some features of typical eukaryotic nucleosome landscape. First, the parasite possesses nucleosome-depleted regions (NDRs) in promoter regions [50, 51]. As in other eukaryotes, a more pronounced NDR correlates with a higher level of transcription, where more open chromatin structure in the promoter leads to a higher level of gene expression. Second, and still under debate among researchers, genic regions show higher levels of nucleosome occupancy as compared to intergenic regions. These results arise from initial nucleosome mapping [51, 52] and FAIRE-seq [53] studies, and are in line with observations in all other eukaryotic genomes [54–56] including *Tetrahymena thermophila* [57], another organism with an AT-rich genome. Some studies dispute this finding and suggest that the more nucleosome-sparse intergenic regions may be caused by preferential digestion of AT-rich regions during nucleosome mapping [50]. Although still controversial, it is possible that with the exception of the telomere ends, the histone variant H2A.Z found ubiquitously throughout intergenic regions of the *Plasmodium* genome generate a weak interaction with the DNA. It has been demonstrated in humans, mice and plants that nucleosomes containing H2A.Z confer lower nucleosome stability compared with other H2A variants [58–60]. In *Plasmodium*, we can speculate that H2A.Z containing nucleosomes could play a chromatin-destabilizing role, which may be important for transcriptional activation in an organism that seems to lack a large amount of specific TFs.

While classical eukaryotic features of nucleosome positioning are clearly conserved in *P. falciparum*, some traits of parasite chromatin are known to be divergent from other eukaryotes while others remain controversial in the field. First, evidence has shown that the strongly positioned +1 nucleosome that is found immediately downstream of the TSS in other eukaryotes is missing in *P. falciparum* [51, 61], with strongly positioned nucleosomes instead observed at the beginnings and ends of coding regions [61]. However, it is important to highlight that more recent studies displayed a conserved +1 nucleosome relative to TSS locations that arose from RNA-seq data [50] or modified CAGE (cap analysis of gene expression) [62]. If present, the +1 nucleo-

some may be more weakly positioned than in other eukaryotes [50]. In fact, a more recent machine learning algorithm incorporating several published epigenetic data sets demonstrated that epigenetic features and nucleosome positioning at the start codons outperformed TSS for predicting transcription in *P. falciparum* [63]. Second, otherwise conflicting nucleosome studies concur that the arrays of nucleosomes within genes display a less phased and more random distribution than is typically observed in eukaryotes (Figure 1C) [50, 51]. It has been proposed that the high AT-content of the parasite genome may cause nucleosomes to bind at preferential locations rather than at fixed distances from other nucleosomes, and that the especially AT-rich intergenic regions cause strong nucleosome positioning at the beginnings and ends of coding regions by acting as barriers [51]. Third, as stated above, histone variants H2A.Z and H2B.Z are found ubiquitously throughout intergenic regions of the core chromosomes [64, 65], rather than simply marking active promoters to poise genes for transcription as in other eukaryotes [19–21]. As their levels positively correlate with AT content [65], it is possible that these variants may have a specialized function for promoting nucleosome deposition in AT-rich regions. H2A.Z and H2B.Z have also been found to be prominently acetylated during the asexual replication cycle [66]. These acetylations may also be important for nucleosome stability and chromatin organization in the AT-rich intergenic regions of the parasite genome.

Another area of debate pertaining to parasite nucleosome landscape is the variation of nucleosome levels during the progression of the parasite life cycle. Evidence suggests that global nucleosome levels drop during the trophozoite stage to allow the transcription of thousands of genes, then rise again as the life cycle progresses through the schizont stage toward egress of merozoites and invasion of new red blood cells [51, 53]. In this model, the weak correlation observed between changes in nucleosome positioning versus mRNA steady-state transcript levels for at least 30% of parasite genes [53, 67] could be explained by mechanisms regulating gene expression at the post-transcriptional level [68, 69]. This hypothesis is supported by the presence of a large number of mRNA-binding proteins identified and validated in the parasite [70, 71] as well as mechanisms of gene regulation identified at the translational level [69]. As opposed to global nucleosome depletion tied to large-scale transcriptional activation, other studies propose that changes in nucleosome positioning at regulatory regions generally correlate with the amount of transcription observed at the mRNA steady-state level [50]. In addition, recent nascent transcript capture using 4-thiouracil incorporation via pyrimidine salvage [72] as well as ATAC-seq (Assay for Transposase-Accessible Chromatin coupled to next-generation sequencing) [73] experiments during the intra-erythrocytic cycle have displayed a dynamic change of ATAC-seq signal that correlates with the cascade of stage-specific expression of the associated genes [74, 75]. It is highly possible that discrepancies observed between studies could be explained by cell cycle timing and data normalization. Normalization by parasitemia or number of nuclei largely reconciles the differences in these opposing datasets. A recent adaptation of single-cell RNA-sequencing (scRNA-seq) experiments was able to resolve some of these issues [76]. As scRNA-seq produces transcriptomic profiles for multiple individual cells, the authors were able to observe sharp transcriptional transitions over the asexual life cycle, which was previously thought to be a continuous process described as a 'cascade of transcripts' [4, 72]. These results further confirmed discrete transcriptional signatures observed using nascent RNA sequencing technology [67] that correlate with sharp changes in nucleosome positioning and chromatin structure and suggest that gene expression throughout parasite development is not as continuous as commonly thought. In addition, a wide variety of complementary approaches confirm that nucleosome occupancy changes throughout the parasite life cycle. These include Western blots [68], mass spectrometry [51, 66, 77], MNase-seq, FAIRE-seq [53] and ChIP-seq [51] experiments. Furthermore, ATAC-seq results have also validated that the highest number of promoter peaks representing accessible chromatin regions are found during the trophozoite stage [74]. Collectively, all these datasets propose a model for gene regulation where nucleosome eviction, open chromatin structure and the limited number of validated TFs drive the active transcriptional state observed at the trophozoite stage, followed by an increase in nucleosome levels and reduction in gene expression during the schizont stage. At this later stage, parasite-specific TFs (AP2), as well as histone PTMs are likely regulating transcription at the initiation level in a more classical manner.

P. falciparum epigenetic regulation

Along with the genome-wide nucleosome landscape, other layers of epigenetic regulation such as histone modifications correlate with gene expression in *P. falciparum*. With only a small number of TFs to regulate the predicted 5472 protein-coding genes in the parasite (genome version: 06-18-2015, http://plasmodb.org/plasmo), changes in histone modifications have been demonstrated to play significant roles in controlling gene expression. This is particularly true for virulence genes involved in immune evasion (*var* genes). *Var* genes encode variants of erythrocyte membrane protein 1 (PfEMP1), a protein exported to the surface of the infected erythrocyte. PfEMP1 plays a key role in cytoadherence of the red blood cell (RBC) and immune evasion inside the human host [78]. Approximately 60 *var* genes are present in the parasite genome, but only one is expressed at a given time, and switching expression creates antigenic variation and allows the parasite to evade the host immune system [79–81]. The mechanisms regulating *var* gene expression have been thoroughly studied *in vitro*, and results from these studies have unveiled complex epigenetic features.

The 59 silenced *var* genes, and other associated silenced genes like PfAP2-G, the gametocyte stage-promoting TF, are marked by the repressive histone modification H3K9me3 and PfHP1 [41, 44, 46, 47]. PfHP1 binds to H3K9me3 and is essential in maintaining repressive heterochromatin. The absence of PfHP1 results in simultaneous expression of nearly all *var* genes as well as cell-cycle arrest during the asexual cycle and an abnormally high rate of sexual differentiation due to derepression of PfAP2-G [82]. *Var* genes have also been shown to cluster together in one or more repressive regions at the nuclear periphery of the parasite [42–44, 47, 53, 83]. Other proteins have also been demonstrated as playing key roles in maintaining the heterochromatin cluster(s), including nuclear class II protein PfHDA2, which has been validated as essential for silencing *var* genes and PfAP2-G, the TF critical for sexual differentiation [84]. Other chromatin-modifying enzymes include HKMTs such as PfSET2, an enzyme which marks nucleosomes with H3K36me3 and was determined as essential for *var* gene repression [85, 86]. Disruption of PfSET2 or its interaction with RNA pol II results in expression of nearly the entire *var* gene family. Finally, the sirtuin proteins PfSIR2A or PfSIR2B have been shown to have a role in chromatin condensation and *var* gene silencing through HDAC

activity. Initial studies conducted in the 3D7 strain showed that the loss of *PfSir2a* causes de-silencing of multiple *var* genes [87], while the loss of *PfSir2b* causes a modest loss of silencing [87, 88]. However, more recent evidence suggests that monoallelic *var* expression in other lab strains is much less sensitive to the loss of these sirtuin proteins [89]. Complementary studies of the initial PfSIR2A knockout strain demonstrated extensive chromosome rearrangements including large deletions in the genome. This latest result created uncertainty about the exact role of these proteins *in vivo*. It is most likely that other component(s) of the genome depleted in the initial knockout experiment are in fact responsible for the loss of regulation of *var* expression in 3D7. Further experiments will be required to identify the presence of such additional regulatory elements.

In wild-type parasites, the single active *var* gene is transcribed at the late ring and trophozoite stages and is prominently marked by H3K4me3, H3K9ac and H3K27ac [44, 83, 87, 90]. The *Plasmodium* histone lysine methyltransferase (HKMT) PfSET10 seems to associate with the active *var* gene and is likely responsible for transcription and epigenetic memory by keeping this particular *var* gene poised for activation in daughter parasites (Figure 2A) [91]. Finally, predominantly euchromatic marker H4K8ac, found to be one of the most sensitive modifications to HDAC inhibitors in *Plasmodium*, also functions to induce expression of the active *var* gene [45]. The many ties between histone-modifying proteins and the expression and repression of *var* genes illustrate that chromatin structure is the most prominent method for the parasite to control mechanisms involved in immune evasion.

Epigenetics of sexual differentiation

With each round of asexual replication, a fraction of parasites commits to sexual differentiation into gametocytes, the stage that is transmitted from human to mosquito. TF PfAP2-G, located on chromosome 12, is well-established as a master regulator of this pathway, positively regulating a set of genes that promote gametocytogenesis [7, 8]. In asexual parasites, this gene is silenced by H3K9me3 and PfHP1 and is found to colocalize with repressed clonally variant gene families such as *var* [44, 46]. Experimental PfHP1 deletion is sufficient to activate PfAP2-G and increase the rate of gametocyte production *in vitro* [82]. Recently, GDV1 (gametocyte development 1) protein was identified as an upstream activator of sexual differentiation [92]. GDV1 functions by evicting PfHP1 from H3K9me3 sites, and is itself repressed during the asexual cycle by an antisense multi-exon lncRNA transcribed from the *gdv1* locus (Figure 2B). Although we know that GDV1 and the gametocytogenesis pathway are activated by environmental conditions [92], it is yet unknown how this lncRNA transduces the signal from these conditions and ceases to repress GDV1.

Invasion genes

When merozoites are released from a human RBC after schizogony, they must invade new erythrocytes by means of ligand-receptor interactions at the red blood cell membrane. Some of these interactions are essential, with knockout of the ligands involved rendering invasion impossible, but others are disposable and variant between parasite lines [93–95]. Some of these invasion-related multigene families with variably expressed members include *eba*, *rhoph1/clag*, *acbp* and *PfRH* [93, 95–97]. Some members of these gene families can exist in either an activated or silenced state, with clonally transmitted silencing that occurs without changes in DNA sequence, suggesting epigenetic regulation [96]. As an example, 95% similar solute transporters *clag3.1* and *clag3.2* are expressed in a mutually exclusively manner, with one gene being silenced and the other expressed throughout a clonal line [96]. Selection of parasites with blasticidin S leads to suppression of both of these proteins, lowering host cell permeability and reducing the amount of drug that can reach the parasite [98–100]. This represents the first known example of an epigenetic drug resistance mechanism in malaria parasites [98]. PfRH4, another gene that can be activated or silenced in a parasite line, confers the ability to invade RBCs that do not express sialic acid as a receptor [101]. Its activation is associated with acetylation of H3 and H4, while its repression correlates with H3K4me3 in the 5′ untranslated region.

Recently, researchers have found two proteins to have large-scale effects on invasion gene transcription. A bromodomain protein, PfBDP1, positively regulates specific invasion genes at the schizont stage by binding to acetylated histone H3 (H3K9ac and H3K14ac) at the TSSs [102]. The importance of PfBDP1 in invasion gene expression has been demonstrated by a conditional knockdown experiment, which leads to defects in growth and invasion [102]. TF PfAP2-I has also been demonstrated as one of the main components driving transcription of invasion genes. PfAP2-I binds to similar upstream motifs and likely forms a complex with PfBDP1 [103]. In this particular context, invasion genes are regulated by more ‘classical’ molecular features that combine *trans*-acting factors with epigenetics to create a complex network of invasion gene expression (Figure 2C).

Long noncoding RNAs

In *P. falciparum,* as in other eukaryotes, lncRNAs are likely to affect gene expression in many ways. One of these is regulation of monoallelic *var* gene expression, in a mechanism that shares traits with mammalian X-inactivation processes. Each *var* gene contains two exons, and the intron between them houses a bidirectional promoter from which two lncRNAs are transcribed [104, 105]. Both of these lncRNAs are incorporated into chromatin after being capped but not polyadenylated. Evidence shows that the two lncRNAs likely have opposite functions. A silent *var* gene is activated by the expression of its specific antisense transcript while interference with these antisense lncRNAs leads to transcriptional repression of the active *var* gene, which triggers *var* gene switching. As a whole, the bidirectional promoter within the *var* intron acts as a silencer when expressing the sense lncRNAs and as an enhancer when expressing the antisense transcripts. Because these antisense lncRNAs were detected at multiple examined *var* genes [106], they have been proposed to contribute to the maintenance of epigenetic memory by recruiting chromatin-modifying enzymes that drive specific histone marks and imprint the active locus for the next cycles and ensure a very slow switch rate [104]. Gene regulation by lncRNAs has also been suggested to control sexual commitment in a mechanism reminiscent of gametogenesis in yeasts [92]. As mentioned previously, GDV1, a protein controlled by environmental triggers, was showed to induce sexual conversion via a *gdv1* antisense RNA. *gdv1* lncRNA, is an example of a noncoding RNA regulating the gene at the locus from which the RNA is transcribed [92]. Other lncRNAs in the parasite have been identified, including a family of homologous lncRNAs transcribed from the telomere-associated repetitive elements (TAREs) [107, 108].

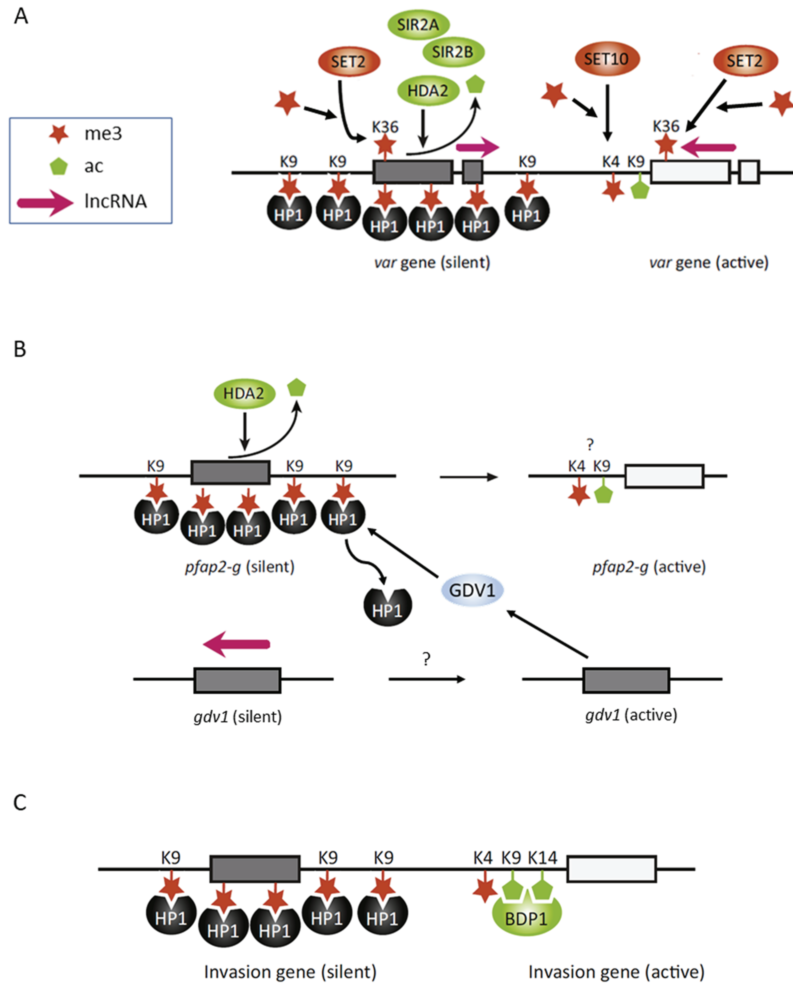

Figure 2. Epigenetic regulation of notable genes and clonally variant gene families in *P. falciparum*. (A) All *var* genes except one are epigenetically silenced at any given time. The silent *var* genes cluster together in a heterochromatic region marked by H3K9me3, to which HP1 binds. The active *var* gene, located separately, is associated with active histone marks H3K4me3 and H3K9ac. (B) PfAP2-G, the master regulator of gametocytogenesis, is epigenetically silenced during the asexual cycle, clustering together with the silent *var* genes. HDA2 helps to maintain this silencing. The transition of AP2-G to an active state during gametocytogenesis is mediated by GDV1, which antagonizes HP1. GDV1 is silenced by an antisense lncRNA transcribed from the gene locus, until the transition to the gametocyte stage. (C) Some invasion genes and gene families display clonally variant expression, suggesting epigenetic regulation. PfBDP1 binds to histone acetylations and promotes invasion gene action in a manner akin to a classical TF. These factors lead to a complex network of invasion gene expression.

Genes encoding these lncRNA-TAREs are found on at least 15 of the 28 *P. falciparum* chromosome ends and are coordinately expressed, with strong induction between DNA replication and cell division cycles [107]. This suggests a role in telomere replication and structural maintenance, perhaps analogous to the long telomeric repeat-containing RNA lncRNAs in mammals [109]. Furthermore, motif search analysis performed on these lncRNA-TARE identified palindromic subtelomeric *var* promoter element 2, suggesting that they may interact with the *var* gene promoter and regulate gene expression by directly or indirectly recruiting histone modifying enzymes when needed [107].

3D nuclear architecture in the life cycle of *P. falciparum*

Examination of the 3D traits of *P. falciparum* chromatin represents another avenue for investigation of how parasite chromatin structure regulates transcription and what specific changes contribute to differential expression in separate life cycle stages.

Experimental tools to investigate 3D chromatin structure

Since the advent of chromosome conformation capture (3C) in 2002 [110], this method and others based on it have been used to investigate chromatin spatial organization in the nucleus and how this relates to expression and function of genes [111]. The original 3C technique can be used to characterize the frequency of physical interaction between two genomic loci (one-versus-one) [110]. The protocol involves *in situ* crosslinking of chromatin using formaldehyde, digestion with a restriction enzyme, re-ligation of DNA ends in close proximity and finally quantitative PCR with primers designed for the two loci under investigation [110, 111]. Later techniques built upon the 3C foundation successfully increased the number of loci under investigation. Chromosome conformation capture on chip (4C), or its variant utilizing next-generation sequencing (4C-seq) can be used to examine all loci that interact with one particular locus of interest (one-versus-all) [112]. Further building upon this foundation, chromosome conformation capture carbon copy (5C) allows

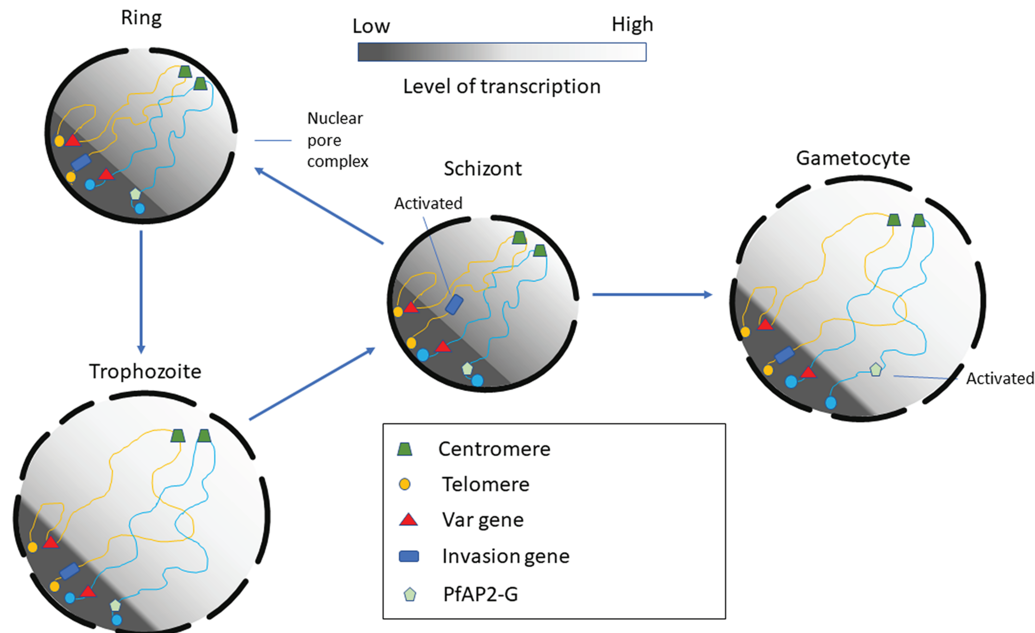

Figure 3. Changes in 3D nuclear structure throughout the life cycle of *P. falciparum*. A region of low gene expression, housing the telomeres and seen at all asexual stages as well as the gametocyte stage, associates with distinct genes at different life cycle stages. During the schizont stage immediately preceding invasion of new red blood cells, invasion genes are seen to leave the repressive region. In gametocytes, PfAP2-G dissociates from the region and becomes active, promoting expression of gametocyte-specific genes. The physical properties of the nucleus change throughout the life cycle as well. During the trophozoite stage, the nucleus enlarges and more nuclear pores are seen, most likely reflecting the high level transcriptional activity at this stage. The nucleus at all stages displays a gradient of expression from a low point where telomeres cluster together to a high point containing the centromeres.

determination of interactions in a 'many-versus-many' fashion by employing multiplex PCR targeting restriction sites in regions of interest in the genome [113]. Finally, the greater availability of next-generation sequencing (NGS) has allowed the creation of 'all-versus-all' methods including Hi-C, which has been used to study 3D chromatin structure of entire genomes in the nucleus [114]. These methods allow 3D modeling based on incidences of physical interactions between loci. The Hi-C technique makes use of biotin-labeled nucleotides at restriction ends to allow pulldown of interaction regions, with the results read out by NGS [114]. Hi-C has allowed investigation of the extent to which chromosomes cluster together in the nucleus, as well as regions that physically interact despite their genomic locations on separate chromosomes [111].

Traits of 3D nuclear architecture in *P. falciparum*

Use of the 3C-based methods to examine the nuclear structure of eukaryotes including *P. falciparum* built upon earlier results using microscopy with immunofluorescence and fluorescence *in situ* hybridization, which have long been used to explore 3D nuclear architecture and chromosome territories in eukaryotes such as human [115, 116]. Use of these microscopy-based techniques on the parasite revealed repressive regions in the subtelomeres as well as some internal loci [42, 48]. The identified heterochromatic regions contain genes involved in antigenic variation such as the *var* gene family and invasion genes. These results were later validated by Hi-C, which was used to capture intra- and interchromosomal interactions in a genome-wide manner [43, 117].

Hi-C experiments have shown a generally simple nuclear organization in *P. falciparum*, along with specific surprisingly complex traits similar to those seen in higher eukaryotes. Pointing to its general simplicity, the parasite genome architecture does not seem to contain well-defined topologically associating domains like those found in human, mouse and *Drosophila* nuclei [118–120]. However, the *P. falciparum* nucleus does exhibit more complex features, which are largely associated with virulence gene regulation. Hi-C experiments confirmed that the *var* genes colocalize at the periphery of the nucleus and demonstrated that chromatin looping brings internal *var* genes physically close to subtelomeric chromosomal regions (Figure 3) [43]. Clustering of a gene family located on different chromosomes has also been observed in more complex eukaryotes. For example, olfactory receptor gene regulation in mice is conceptually similar to *var* gene regulation, with only one gene out of about 2800 genes being expressed at a given time [43, 121]. As in the parasite, the remaining nonexpressed gene family members colocalize to a heterochromatin compartment enriched in repressive histone modifications [121]. Thus, the general nuclear architecture of *P. falciparum* displays the foundational simplicity expected in a single-celled eukaryotic organism but includes additional features seen in more generally complex eukaryotes.

Another layer of complexity in *P. falciparum* genome architecture involves its distinct changes throughout the parasite's life cycle. Hi-C and advanced microscopy have demonstrated that chromatin structure and the nucleus itself are heavily remodeled during the asexual, sexual and transmission stages of the life cycle, which likely correlates with the changes in transcriptional activity that occur during transitions between these stages [43, 117]. After invading an erythrocyte, the parasite is found in the ring stage. At 18–24 hours post-invasion, a number of changes occur during the transition into the trophozoite stage. The nucleus physically expands and reaches its maximum size and volume at the trophozoite stage [122], as can be seen by Giemsa-stained images of parasites [43]. During this transition,

the number of nuclear pores drastically increases from 3–7 to 12–58. At the trophozoite stage the nuclear pores also display a more uniform distribution around the nucleus [122]. As nuclear pores are associated with regions of active transcription and export of transcripts from the nucleus to the cytoplasm, this arrangement may be necessary for the high transcriptional activity observed at the trophozoite stage using nascent RNA sequencing [67]. Chromatin structure becomes more open at the trophozoite stage, displaying nucleosome eviction and increased frequency of interchromosomal contacts [122], also consistent with a high level of transcription. Then, as the parasite enters schizogony, these changes are reversed. With each nuclear division, pores are distributed so that each daughter cell has fewer pores than the mother cell, reaching as low as 2–6 pores per nucleus in late schizonts. During this process of schizogony, redeposition of nucleosomes occurs, chromosome territories become more clearly defined and chromatin structure has a whole recompacts [122]. Another striking feature of the 3D structure in *Plasmodium* is that gene expression correlates with spatial positioning within the nucleus [43]. Experimental data indicate a gradient of expression across the nucleus, from a repressive center near the telomeres to a transcriptionally permissive center at the centromeres. These results suggest that the parasite genome may encode regulatory information in the 3D position of a gene, in addition to its local epigenetic state (Figure 3).

Specific changes to chromatin in the nucleus also occur during sexual differentiation into gametocytes in preparation for transmission from human to mosquito. During this process, the heterochromatin region expands significantly to include genes not previously associated with the region during the asexual cycle. Expansion of heterochromatin was identified using both Hi-C and H3K9me3 ChIP-seq experiments [92, 117, 123]. Many of the genes interacting with heterochromatin during transmission stages encode proteins that are known to be exported to the surface of the red blood cell and involved in erythrocyte remodeling. Others encode invasion-related proteins that mediate attachment and entry of merozoites into red blood cells [92, 117, 123]. Furthermore, Hi-C experiments have demonstrated that the TF *pfap2-g*, the master regulator of sexual differentiation, no longer colocalizes with the heterochromatin cluster in early stage II/III gametocytes, suggesting that *pfap2-g* physically leaves the repressive center during early gametocytogenesis [117].

Hi-C data also reveal the formation of two superdomains on chromosome 14 during sexual differentiation. This chromosomal bipartite structure has been observed in compacted inactive X (Xi) chromosomes in mammals [124, 125]. In eukaryotic genomes, the boundary region between the two large domains has been associated with a higher level of expression and constraint of the spread of heterochromatin [119, 126]. Thus, it is interesting that the domain boundary is located near or inside the gene encoding serine/threonine protein phosphatase 2A activator (PfPTPA) [117], which blocks the G2/M transition to mitotic division when injected into *Xenopus* oocytes [127]. This would be consistent with sexual-stage parasites, which have exited the blood-stage replication cycle. The domain boundary also lies near the gene encoding ApiAP2 TF PfAP2-O3 [117]. In a transgenic *P. berghei* strain expressing a GFP-tagged version of PfAP2-O3, this protein localized to the nucleus in female gametocytes while being completely absent in male gametocytes and gametes, which supports the idea that the boundary may be present only in females [117].

Large-scale chromatin structure rearrangement was also seen in sporozoites, the form in which the parasite is transmitted from mosquito to human [117]. For example, while erythrocyte invasion genes remained associated with repressed virulence genes, significant long-range interactions were observed between genes related to sporozoite migration to the liver and hepatocyte invasion.

In summary, dynamic nuclear organization seems to orchestrate gene expression differences between life cycles stages in *P. falciparum*, highlighting the importance of understanding the molecular players behind this process.

Conclusion

Collectively, the results detailed above demonstrate that epigenetics and chromatin structure, at least partially, mediate genome-wide changes in gene expression throughout the *P. falciparum* life cycle. This may compensate for the lack of TFs identified and validated in the parasite genome. Because chromatin modification and changes in nuclear organization have such an impact on gene expression and life cycle progression, exploration of the molecular components regulating these processes could lead to discovery of proteins and/or lncRNAs that are crucial for the survival of the parasite. Additional research into this field will be imperative in order to discover parasite-specific targets for novel therapeutic strategies that could lessen the global burden caused by malaria.

Key Points

- Epigenetic mechanisms are critical in regulating the complex gene expression program of *P. falciparum*.
- While many aspects of chromatin structure in eukaryotes are conserved in the parasite, some specific features differ, likely to compensate for the extreme AT-content of the *P. falciparum* genome.
- The parasite has evolved specific epigenetic features, most likely to facilitate gene expression of parasite-specific genes including clonally variant gene families, invasion genes and the genes responsible for promoting sexual differentiation in parasite such as PfAP2-G.
- Investigation of the 3-dimensional structure of the nucleus by chromosome conformation capture (3C)-based methods corroborates earlier results and yields novel insights into specific chromatin structure throughout the parasite life cycle stages.

Acknowledgements

We would like to thank Gayani Batugedara for her effort in reviewing and editing this manuscript.

Funding

This work was supported by the National Institutes of Allergy and Infectious Diseases and the National Institutes of Health (grants R01 AI06775, R01 AI136511 and 1 R21 AI142506-01 to K.L.R.) and the University of California, Riverside (NIFA-Hatch-225935 to K.L.R.).

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