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Epigenetic Regulation and Chromatin Remodeling in Malaria Parasites

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Plasmodium, single cell, epigenetics, lncRNA, long noncoding RNA, chromatin architecture

Abstract

Plasmodium falciparum, the human malaria parasite, infects two hosts and various cell types, inducing distinct morphological and physiological changes in the parasite in response to different environmental conditions. These variations required the parasite to adapt and develop elaborate molecular mechanisms to ensure its spread and transmission. Recent findings have significantly improved our understanding of the regulation of gene expression in *P. falciparum*. Here, we provide an up-to-date overview of technologies used to highlight the transcriptomic adjustments occurring in the parasite throughout its life cycle. We also emphasize the complementary and complex epigenetic mechanisms regulating gene expression in malaria parasites. This review concludes with an outlook on the chromatin architecture, the remodeling systems, and how this 3D genome organization is critical in various biological processes.

Contents

1. INTRODUCTION

Global malaria deaths have gradually declined from 896,000 in 2000 to 558,000 in 2019 (134). This has been possible through an increase in malaria control efforts, including distribution of insecticide-treated mosquito bed nets and systemic insecticide spraying. Recently, RTS,S became the first malaria vaccine to be recommended by the WHO. In a pilot study, 2.3 million doses of the vaccine were administered in three African countries, and an approximately 30% reduction in severe malaria was observed (134). Even if this vaccine does not provide complete protection, it may serve as a significant contributor to a malaria control program.

Malaria is caused by the protozoan *Plasmodium*, which belongs to the Apicomplexa phylum, a large group of parasites including *Toxoplasma*, *Cryptosporidium*, and *Babesia*. Over 200 species of *Plasmodium* are reported, but only 5 of them can infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. While *P. vivax* is the most widespread, with high prevalence in Southeast Asia and Latin America, *P. falciparum* is most common in Africa and causes the greatest morbidity and mortality worldwide.

Despite the progress made over the last decades, the WHO's *World Malaria Report 2021* (134) documented a 12% increase in malaria-related fatalities occurring in 2020. The majority (68%) of this augmentation seemed to be attributable to service disruptions during the COVID-19 pandemic. This crisis highlights the importance of maintaining a steady and continuous regiment to control malaria. In addition to the COVID-19 challenges, the emergence and spread of resistance to antimalarials are proving to be tremendous threats. The mutations that have been observed in the *pfkelch13* gene are strongly associated with reduced sensitivity to artemisinin,

our most effective and last line of defense against *P. falciparum* infection, and they are spreading dangerously around the world, including in Africa. These concerns reinforce the importance of deciphering the biological mechanisms of *Plasmodium*, including gene regulation, to discover novel potential therapeutic targets.

In this review, we present the transcriptome modulations across the different stages of the parasite life cycle. We also discuss the contribution of diverse regulatory mechanisms in gene expression, from epigenetics to chromatin structure.

Epigenetics: field of study focused on factors controlling gene expression without altering DNA sequence

2. *PLASMODIUM* STAGE-SPECIFIC EXPRESSION PATTERNS

2.1. The *Plasmodium falciparum* Life Cycle

The human malaria parasite has a complex life cycle involving an intermediate human host and the definitive host and vector, the female *Anopheles* mosquito. During a blood meal, a *Plasmodium*-infected mosquito transmits sporozoites from its salivary glands to the human bloodstream. These sporozoites migrate to the liver, invade hepatocytes, and initiate parasite amplification. This asymptomatic pre-erythrocytic phase takes roughly 7–10 days for *P. falciparum*. In all *Plasmodium* species, once the pre-erythrocytic cycle is complete, tens of thousands of infectious merozoites are released in the bloodstream to invade red blood cells. Within an erythrocyte, an immature ring stage develops into a large, transcriptionally and metabolically active trophozoite that can then differentiate into a multinucleated schizont. The schizont can divide into up to 32 new merozoites, which burst out of the erythrocyte to reinfect new red blood cells. This rupture is associated with clinical symptoms, and most current treatments target this phase. The erythrocyte invasions are repeated continuously with the new generations of merozoites that perpetuate this asexual cycle. During the intraerythrocytic developmental cycle (IDC), a proportion of parasites differentiate into male and female gametocytes. In *P. falciparum*, this gametocytogenesis is exceptionally long, with maturation after 9–12 days, and is composed of five morphologically distinct stages (stages I–V). These gametocytes are ingested during a new blood meal by a female *Anopheles*. A zygote forms inside the mosquito's gut; a zygote is formed by fertilization of a male microgamete and a female macrogamete. This zygote becomes a mobile ookinete, which in turn migrates and invades the midgut wall to form an oocyst. The oocyst stage can produce thousands of new sporozoites. To complete and perpetuate the life cycle, these sporozoites migrate to the mosquito's salivary glands and are injected into a human during a subsequent blood meal.

2.2. An Overview of the *Plasmodium falciparum* Genome and Its Gene Expression

In 2002, the 22.8-megabase nuclear genome of a *P. falciparum* 3D7 clone was published: ~5,500 annotated genes along 14 chromosomes (42). This genome is still one of the most AT-rich genomes that have been sequenced, with ~80% and ~90% AT content for coding and intergenic regions, respectively. The publication of the *P. falciparum* genome was the foundation for transcriptomic exploration. Consecutively, DNA microarrays (12, 70) were applied to analyze the transcriptome of *P. falciparum* at different stages of development, including gametocyte and sporozoite stages. They exposed how most of the predicted genes are expressed during the IDC, with an active burst of transcription during trophozoite and gametocyte stages. The genes exhibit a periodic transcriptional pattern with timely expression; for example, the genes involved in invasion are expressed during late schizont development, and some genes are specifically expressed in gametocytes and sporozoites. In 2005, a similar approach applied following sexual differentiation identified a subset of genes highly transcribed and expressed in gametocytes, confirming high correlation between the *Plasmodium* transcriptome and the distinct stages of development (113).

The advent of next-generation sequencing brought about new opportunities to decipher the malaria parasite's transcriptome more thoroughly. *Plasmodium* RNA-seq was first performed in 2010, providing novel understanding of overall global gene expression patterns in asexual stages of *P. falciparum* (87). With this method, previous predictions of gene boundaries and splice sites were corrected and novel transcripts were detected, significantly improving the parasite transcriptome resolution. Additional RNA-seq data sets were subsequently generated for asexual (6, 53, 73, 119, 121, 132), sexual (68, 73), and mosquito (44, 72, 140) stages of *P. falciparum*, providing access to a previously untapped source of knowledge. These high-throughput sequencing efforts contributed to detection of antisense variants, noncoding RNAs (ncRNAs), and stage-specific transcripts and to characterization of mutant lines. In 2018, nascent mRNAs were explored by 4-thiouracil incorporation, yielding a high-resolution RNA transcriptome data set for the IDC of *P. falciparum* (88). Sex-specific genes were also identified in *P. falciparum* and *P. berghei*, a rodent malaria species, with NIMA-related kinase 4 (NEK4), CCp1, CCp3, and G377 described as markers for female gametocytes while NEK1, CDP4, and MAP2 kinases were found to be enriched in male gametocytes (65, 68) (**Figure 1**).

Host–parasite interactions are critical to fully understand malaria infection and adaptation within the human host. Although dual RNA-seq allows simultaneous capture of host and pathogen transcriptomes, this powerful strategy has been used only sparingly in *Plasmodium*. It was applied in the analysis of infected and uninfected hepatocytes to identify mucin-13 as a robust human marker of *Plasmodium*-hepatic infection, regardless of the species tested (67). Despite its sporadic application in malaria research, dual RNA-seq delivers an attractive opportunity to gain knowledge on parasite and host cell interactions in parallel that is not yet available through alternative platforms.

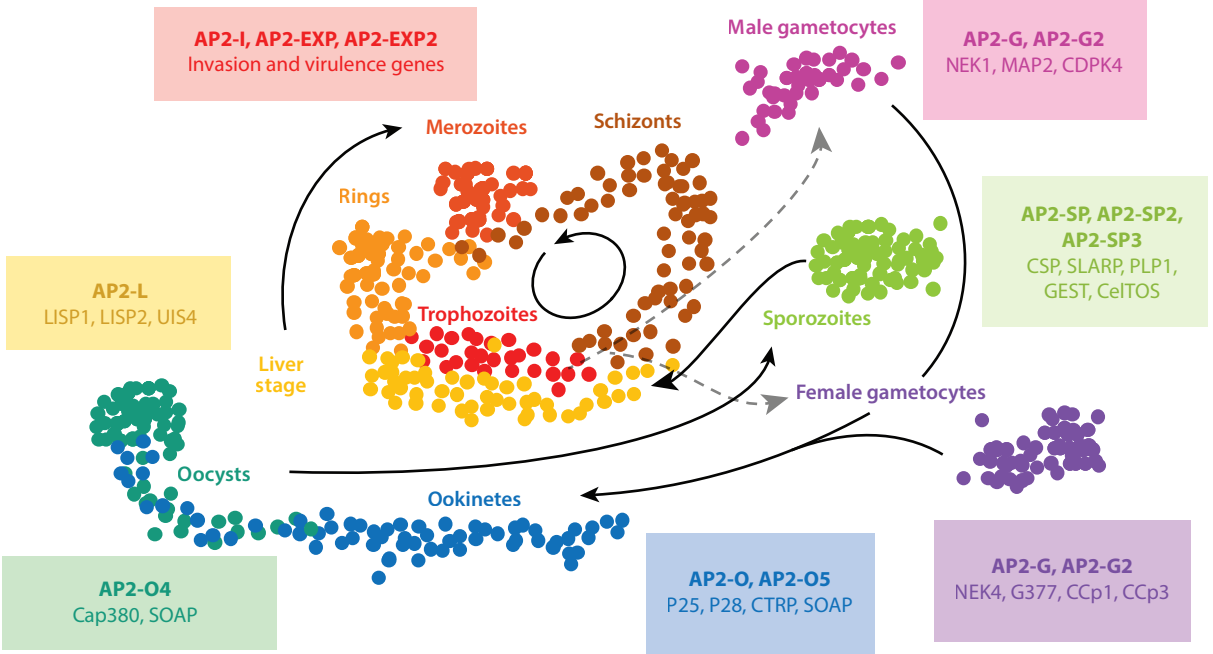

Figure 1

Model of single-cell transcriptomes across the *Plasmodium* life cycle. Cells are colored according to their distinct stage of development. Master ApiAP2 transcription factors are in bold font along with stage-specific or characteristic markers expressed at the particular stages. The model is based on the single-cell atlas of *P. berghei* parasites (55). Abbreviation: ApiAP2, apicomplexan Apetala2.

Recently, single-cell sequencing technologies have emerged as the state-of-the-art platforms for transcriptomic study. Single-cell RNA-seq (scRNA-seq) provides an unbiased cell-to-cell level of RNA transcript detection at a resolution that is unprecedented to date. This technology has revealed previously uncharacterized and rare phenotypes hidden by classical bulk RNA-seq. scRNA-seq was applied to different morphological stages of *P. falciparum*, including asexual (85, 102), sexual (85, 93, 127), and mosquito (83, 101) stages (**Figure 1**). These studies have, among other things, assessed the sex-specific markers of gametocytes (102, 127) and described transcriptional signatures among committed and sexual stages (13, 85, 93). Moreover, additional regulators involved in sexual commitment were identified, such as transcription factors (TFs), SNF2 helicases, and histone-modifying enzymes (93). scRNA-seq data sets and computational methods provide the opportunity to resolve (pseudo)trajectories and deduce the developmental dynamics of the pathogen down to individual cells. Such models were obtained for asexual (102) and mosquito (83, 101) stages of the parasite cell cycle, which provided a more insightful and continuous transcriptomic repertoire instead of the distinct snapshots derived through conventional RNA-seq techniques. Thus far, only the entire life cycle of *P. berghei* has been fully completed and published (55). The authors of this paper generated single-cell transcriptomes from ten stages and detected 5,156 genes divided into 20 clusters. They also compared the blood stage trajectories of *P. falciparum*, *P. knowlesi*, and *P. berghei*. Despite the vastly distinct hosts and IDC periods, their transcriptional activities had an overall pattern. Single-cell technology is also a powerful tool to analyze clinical samples (55) or study the impact of environment changes. *Plasmodium* parasite cultures incubated at 40°C presented a global decrease of transcription and greater transcriptional heterogeneity when compared to control parasites, especially for genes involved in gametocytogenesis and stress response (99).

Overall, the plethora of technologies, optimized over time, have made it possible to decipher the transcriptomes of *Plasmodium* and identify specific transcriptional signatures (**Figure 1**). The Malaria Cell Atlas, managed by the Wellcome Sanger Institute, has already provided an open access platform for single-cell transcriptomics data sets across different parasite cell cycle stages of *P. falciparum*, *P. knowlesi*, and *P. berghei* (**https://www.sanger.ac.uk/tool/mca/mca**). This project will facilitate the global understanding of transcriptional regulation and benefit the malaria research community.

2.3. Transcription Factors and Their Role in *Plasmodium* Gene Regulation

In eukaryotic cells, TFs are key regulators of transcriptional activity. Roughly 1,600 human TFs have been described (66). As described above, *Plasmodium* presents some specific transcriptional signatures suggesting a fine-tuned and well-controlled regulation.

RNA polymerase II is responsible for the transcription of messenger RNAs along with several small nuclear RNAs (**Figure 2**). The polymerase requires general TFs to form a multiprotein complex that assembles onto targeted promoters. Although several of these partners have been identified in *P. falciparum*, the lack of clear orthologs based on homology of critical components found in other eukaryotic lineages suggests that alternative mechanisms may be implicated in the parasite transcriptional machinery (21). For example, recruitment of the preinitiation complex, composed of general TFs and RNA polymerase II, is independent of transcriptional status and histone acetylation in *P. falciparum*, in contrast to the classical eukaryotic model (45). One chromatin immunoprecipitation (ChIP)-on-chip–type experiment demonstrated how RNA polymerase II is bound to promoters in early and late asexual stages (97). During this early phase, the polymerase is engaged with promoter regions yet prevented from further transcriptional elongation. GRO-seq analysis, capturing nascent RNAs, confirmed this pausing, which could contribute to gene regulation and the timely transcriptional burst found in the trophozoite stages (77).

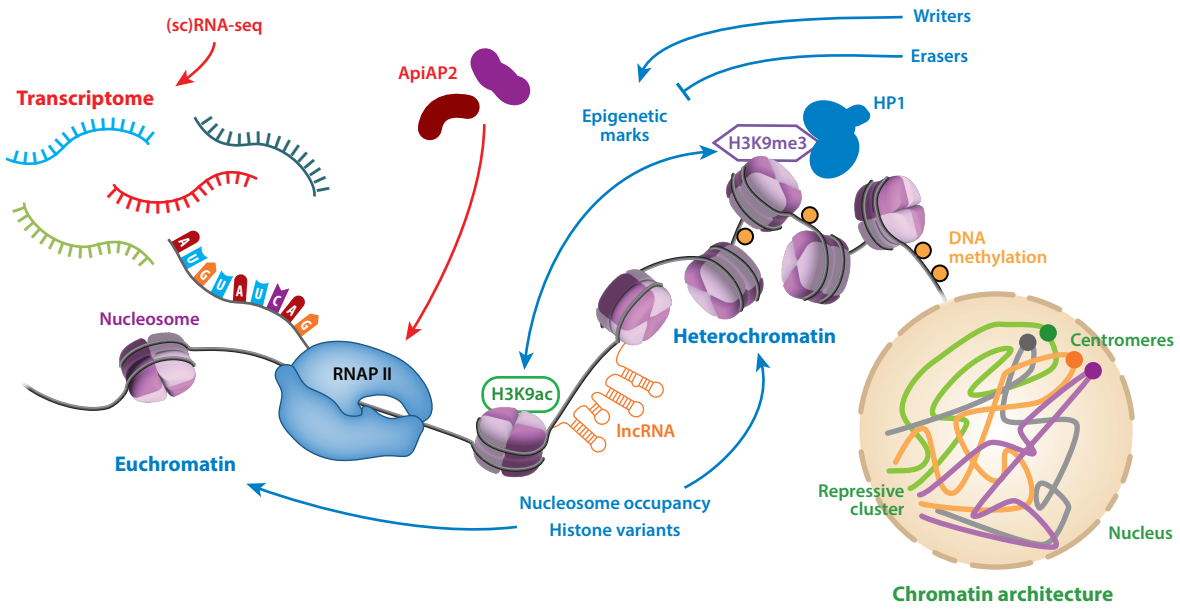

Figure 2

Epigenetic mechanisms in *Plasmodium*. RNA-sequencing platforms (*red*) contribute to the identification of distinct transcriptomic signatures across the different life stages of *Plasmodium*. These gene expression patterns define cell function(s) and are under the control of master ApiAP2 transcription factors. Nucleosome occupancy and histone modifications (*blue*) are major factors regulating chromatin condensation. Other epigenetic mechanisms include lncRNAs and DNA methylation patterns (*orange*). Finally, the overall chromatin landscape is well organized, forming a repressive cluster including telomeres and *var* genes positioned at opposing poles of centromeres within the nucleus (*green*). Abbreviations: ApiAP2, apicomplexan Apetala2; HP1, heterochromatin protein 1; lncRNA, long noncoding RNA; RNAP, RNA polymerase; scRNA-seq, single-cell RNA sequencing.

Besides general TFs, the transcriptional machinery is also dependent on specific TFs, required for the recruitment of chromatin-remodeling and chromatin-modifying enzymes. In 2004, a comparative analysis showed a ratio of one TF per 800 genes in *Plasmodium* (compare to one TF per 29 genes in *Saccharomyces cerevisiae*), indicating a relative paucity of TFs (120). Subsequently, the main specific family of TFs in apicomplexan parasites was discovered (3). These proteins contain Apetala2 (AP2) domains, originally described in plants as DNA-binding domains, and about 20–27 members of this apicomplexan AP2 (ApiAP2) family are highly conserved across all *Plasmodium* species as well as in other apicomplexans. Each protein contains one to three AP2 domains and recognizes multiple distinct DNA motifs (22).

Different studies have reported several of these AP2 TFs as master regulators (activators or repressors) involved in developmental-stage transition (**Figures 1** and **2**). The most characterized of these ApiAP2 is AP2-G, for which gene deletion resulted in abolishment of gametocytogenesis in *P. falciparum* (60) and in *P. berghei* (41). Conversely, AP2-G2 acts as a global repressor of transmission-specific genes, promoting gametocyte maturation (82, 115, 116, 135, 137). Although gametocytogenesis implies a cascade of multiple ApiAP2 TFs (recently reviewed in Reference 94), certain ApiAP2 TFs such as AP2-G5 (107), AP2-G, and AP2-G2 function as main contributors by regulating hundreds of genes and operating as a transcriptional switch from asexual to sexual programs. Similarly, additional master regulators were described in other stages of *P. berghei*, with AP2-O and AP2-Z shown to be essential for ookinetes (61, 86, 139), AP2-SP for sporozoites (138), and AP2-L for liver stages (56). In 2017, two systematic knockout screens of AP2 TFs in

P. berghei (82) and *P. yoelii* (141) malaria models infecting rodents confirmed the importance of these previous TFs but also identified new ApiAP2 members fundamental for mosquito stages, such as AP2-O2/O3/O4 for ookinetes and AP2-SP2/SP3 for sporogony.

Although the majority of the ApiAP2 family TFs are associated with developmental-stage transitions, some of them seem to be required for supplementary mechanisms whose biological functions remain elusive. AP2-exp and AP2-exp2 mutants showed upregulation of antigenic variant genes (*rifin*, *stevor*, and *Pfmc-2TM*) and reduction of genes involved in cell remodeling, respectively (79, 109). Different AP2 TFs are also associated with heterochromatin (108), such as AP2-HC (23), while AP2Tel and PfSIP2 were found to be enriched in subtelomeric regions (38, 112).

Recently, machine learning algorithms, used to build predictive models of gene expression, suggested that upstream regulatory AP2-binding sites may play a more limited role in transcriptional regulation during the IDC than previously thought (100). Several hierarchical players are required to establish and maintain the distinct transcriptional signatures across developmental stages in *Plasmodium*.

Antigenic variant genes: multicopy gene families associated with virulence and immune evasion in *Plasmodium*

Heterochromatin: highly condensed chromatin found mostly within telomeres and repressed genes, as opposed to euchromatin, which corresponds to transcriptionally active regions

3. EPIGENETIC REGULATION IN MALARIA PARASITES: THE ROLE OF NUCLEOSOMES AND HISTONES

3.1. Chromatin Organization, Nucleosome Positioning, and Remodeling

In all eukaryotes, nuclear DNA is compacted and organized into chromatin through the binding of nucleosomes. The core unit of the nucleosome is composed of a histone octamer (two copies each of H2A, H2B, H3, and H4) that wraps ~147 DNA base pairs. Nucleosome occupancy and positioning are widely conserved in eukaryotes, including *Plasmodium*. Transcriptionally active gene promoters are characterized by a nucleosome-depleted region located just upstream of the transcription start site. Comparative studies have found that *P. falciparum* presents relatively weak nucleosome positioning at the +1 and +2 sites compared to model organisms (64); however, the parasite also exhibited significantly higher nucleosome occupancy at the start and end of the gene body (19, 92, 131). Regardless of the methods used to elucidate nucleosome positioning and occupancy—spanning from micrococcal nuclease digestion with deep sequencing (MNase-seq) to formaldehyde-assisted isolation of regulatory elements with deep sequencing (FAIRE-seq) to assay for transposase-accessible chromatin with deep sequencing (ATAC-seq)—it is remarkably clear that the chromatin accessibility correlates with mRNA abundance in *Plasmodium* (19, 64, 92, 100, 103, 121, 131). This association confirms that the depletion of these nucleosomes is fundamental to allow access and binding of TFs and diverse DNA-binding (co)factors/proteins. De novo motif searches using the accessible regions of the chromatin identified motifs already associated with specific AP2 TFs (103, 121), such as AP2-I, which plays an essential role for expression of invasion genes (105).

In *Plasmodium*, nucleosome occupancy is dynamic and varies considerably throughout the cell cycle. During the IDC, the overall histone coverage is minimal at trophozoite stages, whereas maximal coverage is detected during late schizont stages. These patterns clearly correlate to the pathogen's transcription activity and chromatin condensation states (19, 92). Additional studies on sexual and mosquito stages could potentially highlight important dynamic nucleosome changes on regulation of stage-specific genes, as already observed for asexual-specific genes (103, 121).

The four core histones can be substituted by histone variants conferring distinct physical properties, dynamics, and alternative functions. In *Plasmodium*, H2A.Z, H2B.Z, H2Bv, CenH3, and H3.3 were identified as histone variants (52, 80, 118). Interestingly, some of them have specific and unique features, such as H2A.Z/H2B.Z, which is associated with intergenic regions

of *P. falciparum* (54, 90), and PfCENH3, shown to be linked with centromeres (52). H2A.Z also seems to be enriched at the transcription start site of the single active *var* gene, suggesting a contribution of this particular histone variant in regulation of this virulence gene family (89, 90).

Although DNA compaction associated with nucleosomes is understood to hinder accessibility of the transcription machinery, the occupancy and positioning of these nucleosomes provide a complementary and dynamic regulatory mechanism crucial to controlling gene expression in malaria parasites.

***var* genes:** multicopy gene family contributing to antigenic variation whose expression is mutually exclusive to expression of a single gene at a time

ChIP-seq (chromatin immunoprecipitation sequencing): technique for identifying the DNA-binding sites of DNA-binding proteins or histone modifications

3.2. Chromatin Organization and Histone Modifications

In addition to the fundamental roles of nucleosome organization, the histone core itself can be subject to a versatile set of reversible epigenetic marks. These reversible posttranslational modifications (PTMs), which include acetylation, methylation, and phosphorylation, occur on histone tails and are distributed along the genome, mainly on promoter regions. In *Plasmodium*, several dozen PTMs have been identified through quantitative mass spectrometry for each histone, including variants, revealing some PTMs unique to the parasite (27, 80, 104, 106, 123). Interestingly, acetylation of H4 seems to occur from the N terminus to C terminus, whereas deacetylation occurs in the opposite direction (106, 123). This model contrasts with other organisms and seems to be a feature unique to *Plasmodium*.

Although the impact on transcriptional activity of these epigenetic modifications depends on global chromatin environments, some of them are considered to be hallmarks of euchromatin or heterochromatin. H3K9me3 and H3K36me3 are associated with inactive promoters and heterochromatin and are found to be mutually exclusive of H3K9ac and H3K4me3, signatures of active transcription (**Figure 2**). H3K9me3 is recognized and bound by heterochromatin protein 1 (HP1), an interaction conserved across eukaryotes, and their coupled role(s) in silencing *var* genes has been well studied in *Plasmodium*. A clear enrichment of H3K9me3 was observed for inactive *var* genes located in the nuclear periphery of the parasite (26, 75, 98, 104). Conversely, the single active *var* gene was shown to be marked by H3K9ac and H3K4 di- and trimethylation (40, 74).

The majority of these studies were performed on asexual stages, with some specific subsets of these modifications observed during distinct stages of the IDC. In ring stages, histones are enriched in H4K20me1 or H3K56me1, whereas trophozoites are associated with H3K9me2 and H3K18me1 (27). Although there is little information about other stages of *P. falciparum*, recent works have unveiled epigenetic modifications throughout different gametocyte stages (27, 110). Various histone marks were identified as stage specific, such as H3K36me2/me3 for early gametocytes or H3K27me1 and H3K36me1 for late gametocytes (27, 29). However, a recent study has revealed some discrepancies in the PTM patterns (110), substantiating the need to validate histone marks identified by mass spectrometry using alternative tools such as ChIP-seq. In addition, the overall layout of histone marks would stand to benefit from comparative studies across *Plasmodium* species. Complementary studies scrutinizing certain epigenetic modifications in sexual and mosquito stages of *P. berghei* (133) and *P. vivax* (84) were published. In this rodent parasite, for example, the H3K9ac mark correlated with the 5′ untranslated region of active genes throughout different stages, except for female gametocytes. This is most likely because mRNAs are stored and translationally repressed at this stage (133).

Most histone PTM studies have been accomplished at single-site resolution, whereas combinational epigenetic modifications co-occurring on histones have long been neglected. Combinatorial associations were clearly identified in asexual and sexual stages of *P. falciparum*, exposing different forms of cross talk between histone modifications that seem to be fundamental for gene regulation and may be stage specific (27, 106, 110, 125). Similarly, ubiquitination and sumoylation are described in various eukaryotes as histone marks, but they remain poorly characterized in malaria

parasites, despite ubiquitinated PfH2B (80, 106, 110, 123) as well as arginine methylation having been reported (110, 125). Although additional studies will be required to decipher the complexity of the histone code, there is no doubt that these modifications are critical regulators of gene expression throughout the life cycle of *P. falciparum*.

3.3. Chromatin Organization and Histone-Modifying Enzymes

As discussed above, epigenetic modifications are essential for gene regulation in *Plasmodium*. The histone PTMs are reversible and themselves under the control of a wide range of enzymes. These proteins, identified as histone-modifying enzymes, are categorized as writers and erasers and respectively deposit and remove epigenetic marks (**Figure 2**).

Methylation of the histone lysines is controlled by the opposing activity of histone lysine methyltransferases (HKMTs) and demethylases (HKDMs) (30). These enzymatic classes are composed of ten SET domain proteins for HKMTs, and three Jumonji C (JmjC) domain–containing proteins and two LSD1s for HKDMs (30, 57). Similarly, histone acetyltransferases, including MYST and GNAT families, along with histone deacetylases (HDACs), composed of HDAC and sirtuin proteins, have antagonistic effects on the acetylation of histone tails (62). The activities of these enzymes were extensively studied in the regulation of *var* genes in *P. falciparum*. PfSETvs has been shown to be a key regulator through deposition of H3K36me3 on inactive *var* genes (57, 124), as well as the sirtuins Sir2A and Sir2B involved in deacetylation and silencing of these virulence genes (33, 122). These complementary enzymatic activities orchestrate the mutually exclusive expression of *var* genes. Apicidin and curcumin, respectively inhibitors of HDACs and histone acetyltransferase PfGCN5, have been shown to drastically alter the epigenome and transcriptome of *P. falciparum* during the IDC (24, 31). Thus, inhibitors of histone-modifying enzymes are considered potent antimalarial compounds retaining efficacy against multiple stages of *P. falciparum* (28).

In addition to histone-modifying enzymes, chromatin-associated factors play a role in the interpretation of epigenomic marks. These reader proteins recognize specific histone modifications and promote the recruitment of chromatin-remodeling complexes and/or transcriptional machinery. Nearly 30 putative readers have been identified and classified in *P. falciparum*, such as bromodomain and zinc finger proteins, among others (53). Although most of these readers remain uncharacterized, others have been documented, such as PHD1, a PHD finger protein. PHD1 showed high affinity to H3K4me2/me3 and mediated the recruitment of ADA2, GCN5, and PF3D7_1402800, subunits of the transcription coactivator SAGA complex (53). In another instance, disruption of PfGCN5 led to drastic changes of the parasite epigenome and transcriptome, and its interaction with AP2-LT suggested assembly of an atypical SAGA complex in *P. falciparum* (81). The TF PfAP2-I coordinates the expression of invasion genes and may be recruited by PfBDP1, a bromodomain reader recognizing acetylated H3 (59, 105). Taken together, these histone-modifying enzymes have proven to be crucial in regulation of histone modifications and warrant further investigation and characterization, as they are promising therapeutic targets.

4. BEYOND HISTONES: ADDITIONAL REGULATORY FACTORS

4.1. Regulation and Function of DNA Methylation

Besides the fundamental role of nucleosome positioning and histone marks in gene expression, epigenetic traits also encompass additional regulatory mediators. DNA methylation is one example and requires direct chemical modification occurring on the DNA. In mammalian genomes, this reaction is catalyzed by DNA methyltransferases, which transfer a methyl group to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC) (46). An additional oxidized

intermediate, 5-hydroxymethylcytosine (5hmC), can also be generated during the demethylation process. Recruitment of readers to these epigenetic marks affects gene repression or hinders the binding of TFs (**Figure 2**). In *Plasmodium*, the contribution of this mechanism remains poorly understood. Although a unique DNA methyltransferase was identified in the parasite, a pioneering study provided a genome-wide map of 5mC distribution (91). Using bisulfite sequencing (BS-seq) technology, the authors detected that 0.58% of the total genomic cytosines were methylated. However, this method does not distinguish between 5mC and 5hmC. More recently, 5mC levels were detected at 0.01–0.02% of the *P. falciparum* genome, while a new modification, 5hmC-like, was identified as predominant, with 0.19–0.38% of total genomic cytosines (50). While additional data will be needed to confirm the presence of these modifications in the genome, they were found in gene bodies during the IDC; however, correlation with gene expression in asexual stages was found for only a small subset of genes. These findings highlight the need for renewed focus on the processes of DNA methylation in *Plasmodium*.

N^6-Methyladenosine (m^6A) has been known to be present mainly in prokaryotic DNA. Its identification in mammalian cells was controversial, and possibly due to bacterial contamination, nonspecific antibodies, and/or technical limitations. In *Plasmodium* a restriction enzyme–based approach identified the presence of m^6A sites at very low levels in the *P. falciparum* genome, suggesting that this modification may be involved in gene regulation (78). Although these studies confirmed the presence of DNA methylation, the biological mechanisms remain to be investigated.

4.2. The Role of Noncoding RNAs in Gene Regulation

Over the last decades evidence has been mounting on the importance of ncRNAs in cell regulation and gene expression. Although they were initially overlooked and regarded as transcriptional noise, the advent of deep RNA-seq platforms provided an unprecedented advantage to uncover the roles of these previously unacknowledged non-protein-coding molecules. They are now a major focus of study and promising targets for drug therapies, vaccine targets, or diagnostic biomarkers.

In eukaryotes, small ncRNAs such as small interfering RNA (siRNA), microRNA (miRNA), and Piwi-interacting RNA (piRNA) average 18 to 200 nucleotides and function primarily in post-transcriptional modification or silencing and degradation processes. Long ncRNAs (lncRNAs), in contrast, are more than 200 nucleotides and vary in structure, forming complex hairpins and loops that dictate their niche epigenetic and regulatory functions. lncRNAs are classified based on their genomic location, transcriptional arrangements, and/or the transcriptomic mechanisms to which they belong. These ncRNAs can be transcribed as sense lncRNA, antisense lncRNA, intronic lncRNA, bidirectional lncRNA, and long-intergenic lncRNA (lincRNA). Regardless of their categories and features, they have been shown to adopt many mRNA-like traits, such as 5′ 7-methylguanosine capping and RNA polymerase II–mediated transcription (95).

In malaria parasites, there is no evidence for the presence of RNA interference mechanisms (miRNAs, siRNAs, piRNAs); nor is there evidence of conventional regulatory mechanisms based on bioinformatics analyses (9). Instead, these protozoans have adapted a higher-than-average repertoire of RNA-binding proteins and lncRNAs that have been implicated in a wide array of regulatory functions (114) (**Figure 2**). Yet, despite the categorical evidence on the diverse functional relevance of ncRNAs in higher eukaryotes, the functional and mechanistic repertoire of these ncRNAs in most apicomplexan parasites remains elusive. The malaria research community has begun to tap into this new aspect of *Plasmodium* biology, and prominent studies have identified hundreds to thousands of *P. falciparum* ncRNAs (15, 16, 25, 96, 136). Recently, a total of 1,768 intergenic lncRNAs have been identified (7). The subcellular localization and stage-specific expression of several of these putative lncRNAs were validated using RNA fluorescence in situ hybridization (RNA-FISH) and scRNA-seq. Additionally, the genome-wide occupancy of several candidate

nuclear lncRNAs was explored using chromatin isolation by RNA purification (ChIRP). ChIRP-seq of candidate lncRNAs revealed that lncRNA occupancy sites within the parasite genome are focal and sequence specific, with a particular enrichment for several parasite-specific gene families, including those involved in pathogenesis, erythrocyte remodeling, and regulation of sexual differentiation. This study also demonstrated that the presence of some of these lncRNAs correlates with changes in gene expression (7). Moreover, disruption of one of these identified lncRNAs, lncRNA-ch14, resulted in a defect during sexual differentiation and development including sexual reproduction, validating the importance of some of these lncRNAs in gene regulation and stage transition. These lncRNAs could be the missing epigenetic regulators in *Plasmodium*. By interacting with DNA, RNA, and proteins, they can modulate chromatin structure by recruiting histone-modifying enzymes and transcription factors and control transcription.

One of the best-studied features of *Plasmodium* is found with the regulation of *var* genes. lncRNAs are implicated in the modulation of antigenic switching of the PfEMP1 variants through transcription of both a sense lncRNA and an antisense lncRNA. These lncRNAs originate from a bidirectional promoter in the *var* intron and are extended to exon I and exon II for antisense and sense lncRNAs, respectively (36). Expression of antisense lncRNAs from a particular silent *var* increases the activation of this specific gene and triggers, in *trans*, this *var* gene switching (1, 58). Recently, thioredoxin peroxidase I was identified to interact and colocalize with the antisense lncRNA, and its knockdown delayed *var* switching, suggesting a redox sensor role of this protein allowing the activation of a *var* gene depending on the nuclear environment (51). However, CRISPR/Cas9 editing of the *var2csa* intron demonstrated that these lncRNAs are not fundamental for activation and inactivation but may instead play a more subtle role in the switching mechanism than previously believed (17). Additional GC-rich ncRNAs are also described to be involved in the regulation of *var* expression (4, 48, 129). Although the means by which these regulatory mechanisms occur are still under debate and require further study, the current findings clearly validate the prominent implications of ncRNAs in the regulation of antigenic variation.

The antisense lncRNA transcribed from the gametocyte development protein 1 (GDV1) has also been shown to play a critical role in regulation of AP2-G. Identified as disrupted in a gametocyte-deficient line, its complementation restored gametocyte production whereas its overexpression led to higher gametocyte production, confirming the fundamental role of GDV1 in sexual development in *P. falciparum* (35). During unfavorable environmental conditions, the nuclear protein GDV1 evicts HP1 from the H3K9me3 on the *ap2-g* promoter, resulting in destabilization of the silencing mechanisms in place (37). With displacement of HP1, chromatin conformational changes result in euchromatin formation and facilitate transcription of *ap2-g*, initiating gametocytogenesis. It was revealed that during asexual parasite development, a five-exon antisense lncRNA transcribed from the *gdv1* downstream locus is responsible for self-regulation and repression of the gene and indirectly of *ap2-g* (15). Additionally, knockout of this lncRNA increased expression of several genes associated with sexual differentiation (*ap2-g*, *dblmsp2*, *pfg14_748*, and *pf3d7_1477400*) (37). Recently, an inducible gametocyte producer line was engineered to overexpress GDV1, leading to a sexual conversion rate of 75%, a striking difference to the 8% found in the control population (11), validating the essentiality of this gene-lncRNA in this mechanism.

Despite the perplexity and debate maintained in the study of ncRNAs, the relative importance of these molecules across parasite development and transmission is undeniable. From pre- and posttranscriptional modifications to chromatin arrangements and gene regulation, ncRNAs have significant therapeutic and diagnostic potential. Advancements in capture technologies and enhanced sequencing platforms have helped dispel much of the mystery surrounding these molecules, yet there remains much to be resolved.

4.3. DNA Secondary Structures and G-Quadruplex

Cytokinesis: a process of cell division in which cytoplasm is divided between the different nuclei forming the daughter cells

DNA is usually a canonical right-handed DNA helix but also has the ability to form various secondary structures such as hairpins and quadruplexes. G-quadruplex (G4) is one such structure and is formed by self-aggregation of four guanines in DNA and RNA. With the AT-rich genome of *P. falciparum*, G4 structures have long been confined to GC-rich repeats found in telomere ends and *var* genes located in subtelomeric regions (117). Recently, prediction algorithms showed enrichment of G4s not only in telomere regions and subtelomeric and internal *var* genes but also in nucleosome-depleted regions (10, 43). The use of pyridostatin, a G4 ligand, altered the *P. falciparum* transcriptome, resulting in the downregulation of 56–60% of genes containing G4s in promoters or exons (43). To our knowledge, only G-strand-binding protein 2 (GBP2) has been characterized as a G4-binding protein in *P. falciparum*. This protein interacted with G4 structures and telomeres in vivo, protected the parasite from pyridostatin treatment, and played a role in *var* gene regulation (34, 49). Although these pioneering studies support the existence of G4 structures and mediators in *P. falciparum*, their indirect or direct role(s) in gene regulation warrants further investigation.

5. NUCLEAR AND CHROMATIN ARCHITECTURE: DYNAMIC STRUCTURAL CHANGES ACROSS THE MALARIA PARASITE LIFE CYCLE

5.1. Nuclear Architecture

As discussed previously, the transcriptional status of malaria parasites is dynamic and controlled by various and interconnected layers of regulatory mechanisms associated with phenotypic and morphological changes. In the ring stage, for example, the genome is compacted and enriched in nucleosomes, with each nucleus encompassing 3 to 7 tightly clustered nuclear pores (2, 19, 130). This condensation hampers the binding of the transcriptional machinery and is consistent with the low transcription activity detected during ring stages. Conversely, the nuclear volume and the number of nuclear pore complexes (NPCs) are significantly increased in trophozoite stages, validating the open chromatin structure and active transcriptional activity (2, 130). These NPCs are roughly 60 in number and seem not to colocalize with HP1 and H3K9me3, two main heterochromatin markers, suggesting they are largely distributed in euchromatin and active regions (32). The number of pores gradually decreases throughout schizogony, with 6–16 NPCs for mid-schizonts and 2–6 for late schizonts, clustering once more to a single location near the euchromatin region (130). At this stage, nucleosomes are repackaged and the chromatin is compacted to facilitate the processes of cytokinesis and reinvasion, leading to a reduced nuclear volume akin to the ring stage.

Yet, little information is known about the nuclear architecture of the parasite for the sexual and mosquito stages. Female gametocytes have been shown to develop a smaller and more compacted nucleus, with transcripts stored and translationally repressed, while male gametocytes, needing to prepare for mitosis and gamete formation, show larger nucleus volumes. In *P. berghei*, nuclear pores are distant from DNA, but *P. falciparum* has morphologically atypical gametocytes, which has hindered the ability to draw reliable conclusions (63). The remarkable plasticity and patterns of nuclear landscapes between *Plasmodium* spp. and within transitional stages warrant further analysis to help describe these complex parasites.

5.2. Chromatin Architecture

The pathogen is also reliant on the overall chromosomal architecture to mechanistically control the spatial proximity between essential genome components such as promotors and enhances

cross talk between regions otherwise located far apart on the same chromosome or on different chromosomes.

These contact points were first recorded during early studies employing FISH, revealing important loci belonging to *var* gene families in the subtelomeric regions of heterologous chromosomes at the nuclear periphery (39, 75). Clustering of the chromosomal telomeres as a repressive center enriched in H3K9me3 and HP1 protein enables interaction and communication between the *var* gene families, crucial for mutual exclusive expression. These perinuclear repressive clusters congregate additional subtelomeric multigene families associated with reinvasion such as *stevor* and *Pfmc-2TM*. Like their *var* gene neighbors, these clonally variant gene families are subject to mutually exclusive expression during the parasite cell cycle (69).

Hi-C: unbiased method to capture the 3D conformation of genomes based on chromatin fixation, enzymatic digestion, proximity ligation, and deep sequencing

The intra- and interchromosomal organizational patterns were further divulged following the development of 3D chromosome capture tools. Chromosome conformation capture coupled with next-generation sequencing technology (Hi-C) provided the opportunity to record and capture important contact loci in an all-versus-all approach and revealed the complex nature of the chromosomal landscape throughout parasite development (**Figure 3**). The unique patterns of

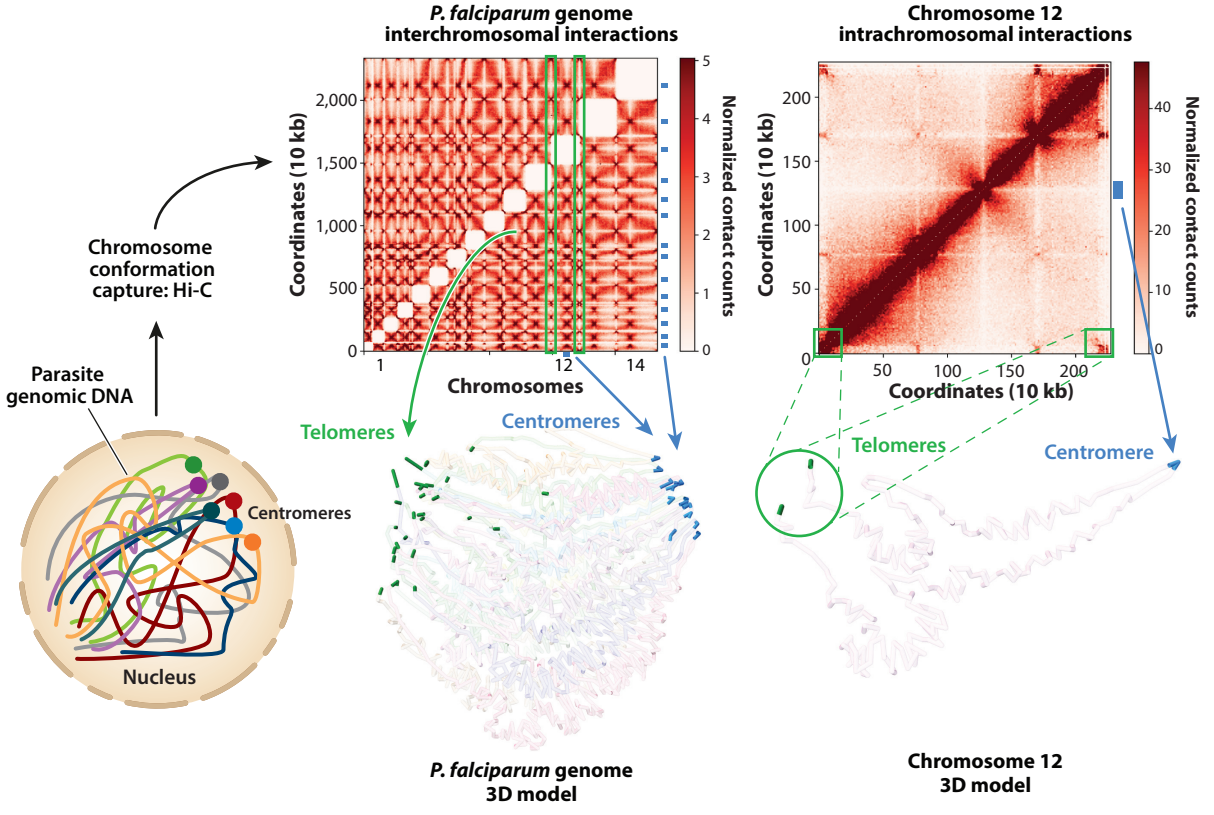

Figure 3

Hi-C data and 3D modeling of the *Plasmodium falciparum* genome. The chromatin conformation of the parasite genome is captured by Hi-C. Analysis of Hi-C data allows the generation of chromosomal contact maps depicting inter- and intrachromosomal interactions. This technique also enables reliable computer modeling of genome 3D structure. Interchromosomal interactions (*left*) between telomeres (*green*) and centromeres (*blue*) of the parasite genome are highlighted. The intrachromosomal interactions and the 3D model of chromosome 12 have been emphasized (*right*).

chromosomal condensation were found to reflect the transcriptomic activity of parasite cell cycle progression. This genomic landscape adopts tight condensation formation throughout the pathogen's early ring stage and during late schizogony, consistent with the low transcriptional activity associated with these parasite stages (2). Conversely, contact maps and 3D models uncovered a more open chromatin throughout the trophozoite stages, when transcription peaks. Hi-C studies revealed the spatiotemporal nature of the parasite's genome whereby centromeres are directed to one end of the nuclear periphery along with discrete telomeric heterochromatin clusters allocated to the opposing end (2, 20, 71). Although no well-defined topologically associating domains were identified in *Plasmodium*, these Hi-C analyses have helped highlight the formation of domain-like structures surrounding the genes involved in antigenic variation bringing the subtelomeric and internal *var* genes loci closer together (**Figure 3**). In *P. falciparum*, a correlation between gene expression and genome organization was observed, with a gradient of expression increasing from the telomere to the centromere (20). A weaker relation was demonstrated for *P. vivax*, *P. knowlesi*, and *P. berghei*, but no correlation was observed for *P. yoelii* and *Babesia microti*.

Distinct changes were also observed for the transmission stages, such as rearrangement of the *ap2-g* locus moving it from the repressive heterochromatin cluster during the asexual stage to the active euchromatin at the early gametocyte stage, which led to its expression (18). Large chromosomal rearrangements were also observed for genes encoding proteins involved in invasion and exported proteins associated with gametocytogenesis and erythrocyte remodeling, indicating their relocation with the repressed virulence genes at the gametocyte stage (18). These interactions were even stronger in sporozoites, confirming their tight repression at this stage. Additional intrachromosomal interactions were detected and involved regions containing genes important for sporozoite migration and invasion such as *csp*, *plp1*, and *gest* (18) (**Figure 1**).

With the advancements in chromatin capture technologies, crucial features have been revealed about the importance of the dynamic nature of the *Plasmodium* chromosomal landscape driving developmental stages of the parasite. Future studies will most likely allow us to learn more about the molecular components regulating chromatin structure in *Plasmodium*.

5.3. Chromatin-Associated Modulators

Chromatin structure is essential for maintaining stage-specific gene expression. This structure is highly dynamic and requires fine organizational regulation by chromatin-associated proteins and lncRNAs and others.

In most metazoan organisms, the nucleus is demarcated by two lipid bilayers and reinforced by a lamina system that serves as a site for mechanosignaling, epigenetic regulation, and nuclear stability (47). Despite the fundamental relevance of this scaffolding hub, evidence of lamina components in many organisms, including *Plasmodium*, has yet to be found. A recent study successfully identified hundreds of chromatin-associated proteins using chromatin enrichment for proteomics (ChEP) (8). The authors also successfully characterized a CRWN-like protein (PF3D7_1325400), a plant-related protein functionally analogous to animal nuclear lamina proteins. Phenotypic analyses of combining mutations demonstrated the essentiality of the CRWN family for the viability of *Arabidopsis* and for proper nuclear organization (128), suggesting a similar role in *Plasmodium*. Findings obtained through immunofluorescence assays revealed this CRWN-like protein was colocalized with H3K9me3, insinuating a role in heterochromatin regulation (8).

High-mobility group box (HMGB) proteins have been a subject of interest, serving critical architectural organization and gene expression roles across the sexual and asexual stages of the parasite. In human cells, these proteins can bind to nucleosomes, lncRNAs, and diverse DNA structures, including G4, and regulate chromatin organization, telomere maintenance, and DNA

repair (126). In *Plasmodium*, four HMGB proteins have been characterized through sequence homology of the conserved HMGB domains (14). Recently, *Pf*HMGB1 was reported to be enriched in the centromeres, and its knockdown increased the distances among the centromeres at the nuclear periphery (76). This local chromatin disorganization also altered telomere clustering, which may explain the complete silencing of all *var* genes upon *Pf*HMGB1 depletion and confirm the critical architectural role of this factor.

lncRNAs also have major roles in chromatin conformational regulation. The most prominent example of lncRNA functional implications lies in the X inactive specific transcript (Xist) expressed in mammalian cells. In eukaryotic cells, Xist regulates gene expression by mediating the inactivation of the X chromosome during zygote development after fertilization (41). In *P. falciparum*, a unique family of roughly 22 lncRNAs labeled as lncRNA-TAREs (telomere-associated repeat elements) was characterized (16). These TERRA (telomere repeat–containing RNA)-like lncRNAs are transcribed by RNA polymerase II from TAREs on almost all chromosome ends (16, 111). lncRNA TERRA is recruited to yeast and mammalian telomeres and is involved in telomerase recruitment, telomere maintenance, and stabilization of protein–telomere interactions (5). lncRNA-TAREs are long intergenic lncRNAs (>4 kb) and may play roles in DNA replication and telomere maintenance (15, 16, 111). lncRNA-TARE-6 consists of degenerate 21-bp repeats and forms a stable secondary structure composed of 12 predicted hairpin loops (111). This lncRNA was shown to bind nuclear proteins and histone H3, suggesting it may be implicated in the recruitment of nuclear factors to regulate chromosomal conformation. Another prominent example is lncRNA-TARE-4. ChIRP-seq experiments demonstrated lncRNA-TARE-4 interaction with most of the subtelomeric regions in a specific manner and revealed two potential motifs associated with lncRNA-TARE-4 binding (7). Taken together, chromatin-associated proteins and lncRNAs have proven to be fundamental regulators of chromatin architecture and warrant further investigation to help decipher at the mechanistic level the role of chromatin structure in gene regulation.

6. CONCLUSION

Collectively, the recent advances discussed in this review demonstrate the array of complex epigenetic mechanisms required to control transcriptional regulation in *Plasmodium*. Nucleosome positioning, TFs, histone modifications, and ncRNAs, among others, participate in a systematic and complementary manner in the dynamics of nuclear organization to coordinate gene regulation throughout parasite development and transmission. Although important formerly underappreciated or unknown regulators have been identified in recent years, our understanding is far from complete.

DISCLOSURE STATEMENT

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