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The role of Long Non-Coding RNAs in malaria parasites

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Abstract

The human malaria parasites, including *Plasmodium falciparum*, persist as a major cause of global morbidity and mortality. The recent stalling of progress toward malaria elimination substantiates a need for novel interventions. Controlled gene expression is central to the parasite's numerous life cycle transformations and adaptation. With few specific transcription factors identified, crucial roles for chromatin states and epigenetics in parasite transcription have become evident. Although many chromatin modifying enzymes are known, less is known about which factors mediate their impacts on transcriptional variation. Like higher eukaryotes, long non-coding RNAs have recently been shown to have integral roles in parasite gene regulation. This review aims to summarize recent developments and key findings on the role of long non-coding RNAs in *P. falciparum*.

Keywords

Malaria; *Plasmodium* ; lncRNAs; Gene regulation; epigenetics; chromatin structure

P. falciparum malaria life cycle and gene regulation

The human malaria parasite *Plasmodium falciparum* prevents progress toward global health equity and causes over half a million annual deaths mainly in infants and pregnant women of sub-Saharan Africa [1]. Over the last few decades, this mortality has been reduced by interventions including artemisinin-based combination therapies, insecticidal nets and rapid diagnostic tests. However, further progress has been hindered by the spread of antimalarial resistance and the lack of highly effective vaccines. Furthermore, the parasite seems to adapt to survive in areas with low transmission and persists as asymptomatic infections that are often missed by diagnostics [2, 3]. To be successful, malaria elimination will require novel diagnostic, therapeutic and intervention strategies.

P. falciparum has a complex life cycle [4, 5]. It infects both human and mosquito hosts and undergoes numerous cellular transformations leading to distinct morphological and physiological changes in response to these altered environmental conditions (Figure 1). The publication of the genome and its annotation in 2002 shed light on the unique

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nature of this pathogen [6]. The 23 megabase *P. falciparum* nuclear genome is about 82% AT-rich. It is organized into 14 chromosomes and encodes approximately 5720 protein coding genes, along with an apicoplast and a mitochondrial genomes [6]. Based on this foundational knowledge, early DNA microarray studies showed that most protein-coding genes are developmentally regulated in transcriptional cascades across the parasite life cycle [7, 8]. More recently, RNA-seq data improved our understanding of transcriptional variation by detecting novel spliced variants and antisense transcripts across the parasite life cycle [9–11]. Single-cell RNA-seq has recently begun to unveil the temporal resolution of transcription and profiles of minority populations [12, 13]. Technological advances and contextual applications of these methods continue to refine our understanding of the importance of transcriptional development and heterogeneity to parasite adaptation for infection, survival and transmission across both hosts [14–16]. Critical to parasite adaptation are genes families described as **clonally variant genes**, or CVGs, (see **Glossary**) that are involved in parasite processes such as immune evasion [10, 11]. Despite this wealth of foundational knowledge, the exact molecular mechanisms that underlie the parasite's tightly controlled regulation of gene expression remain elusive.

While the *P. falciparum* genome encodes an expected number of general transcriptional factors [17, 18], few sequence-specific transcription factors (TFs) have been identified and validated. The apicomplexan apetala2 (ApiAP2) DNA-binding protein family are unique to apicomplexans and share homology with the plant AP2/ethylene response factor integrase DNA binding domain [19]. Several of the 27 ApiAP2 TFs have been demonstrated to be master transcriptional activators and repressors during parasite transitions in the mosquito, liver, IDC and sexual stages [20]. However, little is known about what recruits these master TFs to their DNA binding motifs. Recent machine learning algorithms suggest their role in regulation may be working in conjunction with **epigenetic** factors [21].

Supporting this view, data generated over the years have started to highlight the functional importance of epigenetic mechanisms and **chromatin** to the developmental regulation of parasite transcription [22–25]. **Chromatin conformation capture (Hi-C)** studies in *Plasmodium* species have shown that global chromatin configurations in long-range interaction between subtelomeric and internal chromosome loci correlate with gene expression and form **heterochromatin** clusters that silence most CVGs [26–28]. Hi-C experiments have demonstrated that parasite nuclear compaction loosens along with the increased frequency of nuclear pores and transcriptional activity observed in the highly active trophozoite [29]. N**ucleosome** occupancy studies in *P. falciparum* have also revealed global transformations across the IDC [30]. Gene promoter accessibility correlates to expression levels, with active promoters exhibiting nucleosome depleted regions upstream of transcription start sites (TSSs) [30]. Variant histones also have specific occupancy sites in *P. falciparum*, such as H2A.Z/H2B.Z in euchromatin intergenic regions and PfCENH3 in the centromeric regions [31, 32].

Beyond nucleosome organization, histone cores are subject to reversible **post-translational modifications (PTMs)**, including acetylation, methylation and phosphorylation. Quantitative mass spectrometry studies on each histone identified over 200 PTMs in *P. falciparum* with some unique to the parasite [33]. Certain PTMs are considered distinctive

features of euchromatin or heterochromatin; for example, H3K9me3 and H3K36me3 associate with inactive promoters and heterochromatin and are mutually exclusive of H3K9ac and H3K4me3, signatures of active transcription and euchromatin [34]. While additional studies are needed to unravel the complexity of the histone code, nucleosome positioning and histone PTMs are clearly critical regulators of gene expression as they likely cooperate with ApiAP2 TFs to recruit general transcriptional machinery as needed throughout the parasite life cycle. However, how these parasite factors are specifically recruited to the chromatin remains to be determined. Because gene regulation is critical to *Plasmodium* development and survival, an improved understanding of the underlying mechanisms that regulate gene expression and host-pathogen interaction is likely to open unexplored avenues for novel therapies. In eukaryotes, including *P. falciparum*, long non-coding RNAs have recently been shown to have fundamental roles in gene expression [35–43]. For those reasons, this review focuses on recent advances in our understanding of the roles of lncRNAs in *P. falciparum* gene expression for development, adaptation and survival within its hosts.

long non-coding RNAs in higher eukaryotes

Conserved across eukaryotes, long non-coding RNAs (lncRNAs) are ≥ 200 nucleotide-long transcripts that are typically transcribed by RNA polymerase II but do not encode proteins. Like mRNA, lncRNAs are often capped, spliced and polyadenylated. Based on their genomic origins, lncRNAs are categorized as sense, antisense, bidirectional, intronic and intergenic [44]. As lncRNAs can bind DNAs, RNAs and proteins, their conserved functional range in eukaryotes is expansive [35] (Figure 2). By tethering genomic DNA, lncRNAs can facilitate changes in three-dimensional (3D) chromatin structures and long-range contacts between regulatory factors, like enhancer elements [35]. At the local level, lncRNAs can also regulate the expression of genes via transcriptional interference or as signals for downstream gene expression [35]. Moreover, lncRNAs can regulate gene promoter accessibility by recruiting, guiding or enhancing chromatin remodeling enzymes like histone acetylases and deacetylases [35]. Similarly, lncRNAs can recruit transcriptional factors or influence their activity [35]. LncRNAs can also interact with spliceosomal factors to affect the frequency and efficiency of mRNA splicing [35]. Additionally, some lncRNAs, including circular lncRNAs can act like molecular sponges that mediate available microRNA (miRNA), which bind complementary mRNA to modulate its degradation or translational repression [35]. At the post-transcriptional level, lncRNAs can regulate gene expression by mediating mRNA export and availability for turnover, decay and translation (decoy) [35]. Also in the cytosol, lncRNAs can serve as scaffolds for protein complexes involved in gene expression [35]. By directly interacting with translation factors, lncRNAs can also affect the frequency and efficiency of translation [35].

One of the first lncRNAs discovered in eukaryotes, is the 15–17-kb-long lncRNA X-inactive specific transcript (Xist) that is now known to mediate the allelic exclusion of the inactive X chromosome (Xi) during zygotic development of female placental mammalians [45]. Xist expressed by the Xi recruits chromatin remodeling enzymes to methylate DNA and histones on this chromosome and form **epigenetic memory** for gene silencing. Xist represses genes in *cis* by interacting with the RNA-binding protein SPLEN and multiprotein

Polycomb protein complexes PRC1 and PRC2 which catalyze the PTMs H2AK119ub1 and H3K27me3, respectively [45]. Along with the gain of H3K9me2/3 and H4K20me1, the formation of heritable Xi silencing is marked by the loss of RNA Polymerase II, general transcription factors and activating PTMs like H4ac, H3K9/27ac and H3Kme1/3 [46].

In higher eukaryotes lncRNAs have been linked to diseases ranging from cancers to cardiovascular, neurological and autoimmune diseases and are, therefore, studied for their potential chemotherapeutic, diagnostic, prognostic and predictive values [47]. Like Xist, two other lncRNAs have been shown to interact with PRC1 and PRC2 to silence gene expression: HOX transcript antisense (HOTAIR) and the antisense lncRNA in the INK4 locus (ANRIL) [47]. As the first lncRNA shown to influence transcription in *trans*, HOTAIR regulates skin differentiation and has been linked to numerous epithelial and stromal cancers [47]. ANRIL, on the other hand, upregulates G1 phase cell cycle progression and cancer cell growth by regulating heterochromatin and silencing three important tumor suppressor genes on the nearby sense strand of chromosome 9: the alternative reading frame (ARF) protein p14 and the two cyclin-dependent kinase (CDK) inhibitors p15/CDKN2B and p16/CDKN2A [47].

Also conserved in eukaryotes, telomeric repeat-containing RNAs (TERRAs) are heterogenous lncRNAs that regulate telomere length, possibly by binding DNA to block the reverse transcriptase telomerase or displacing inhibitory proteins [48]. TERRAs also regulate telomere structural integrity by promoting homology dependent repair via their interaction with RAD51 [48]. Lastly, TERRAs are also thought to mediate telomeric heterochromatin by interacting with associated proteins like heterochromatin protein 1 (HP1), the Origin Recognition Complex (ORC) and telomere repeat factors (TRFs) [48].

At the post-transcriptional level, one of the most studied lncRNAs, the metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), is implicated in alternative splicing events by modulating the phosphorylation of serine- and arginine(SR)-rich proteins [47]. A more recent discovery involved the antisense lncRNA to ubiquitin carboxy-terminal hydrolase L1 (AS-Uchl1) that increases UCHL1 protein synthesis at the post-transcriptional level. AS-Uchl1 is regulated by stress signaling pathways and is required for the association of the overlapping sense protein-coding mRNA with active polysomes for translation. Dysregulation of AS-Uchl1 has been implicated in neurodegenerative diseases [35].

long non-coding RNAs in *Plasmodium falciparum*

Although their roles in infectious diseases have only been studied more recently, lncRNAs are beginning to emerge as new players in the development of eukaryotic pathogens within their human hosts, including malaria parasites [36, 37]. In *P. falciparum*, early transcriptomic studies showed the parasite expresses natural antisense lncRNAs across its life cycle [49–56]. Technological advances such as strand-specific and short-read sequencing using nanopore platforms identified novel parasite lncRNAs, including over 1300 circular lncRNAs, and confirmed parasite lncRNAs are regulated developmentally [9, 57–59]. Similarly, profiling of nascent non-coding transcripts, amplification-free sequencing and single molecule real-time (SMRT) long-read sequencing have helped further expand the

repertoire of parasite lncRNAs to > 2,500 candidates [37, 60–62]. Most recently, a leap forward has been made by manually annotating short- and long-read sequencing, unveiling 1,119 novel lncRNAs [63]. In this study, a total of 2,369 lncRNAs expressed in the asexual stages have been identified and categorized as antisense (75%), intergenic (12%), UTR-associated (9%) or sense overlapping an exon or intron (< 2% and < 1%, respectively). Some of these lncRNAs have homology with known RNA families, including those encoding structural RNAs, while others may encode smaller structural RNAs, suggesting lncRNAs may be integral to parasite RNA structure [63]. Although a subset of transcripts identified as lncRNAs may encode small proteins [37, 62, 63], no peptides have been detected for the annotated lncRNAs discussed in this review. Furthermore, while some annotated lncRNAs have been implicated in parasite gene regulation, the functional roles of most parasite lncRNAs have yet to be explored.

A recent study addressed a critical knowledge gap in *P. falciparum* lncRNA biology – where lncRNAs were identified as either enriched in the nuclear or cytoplasmic cellular fraction and, in accordance with conserved functions in higher eukaryotes, more likely involved either in epigenetic or translational regulation, respectively [43]. Of the 1,768 candidate intergenic lncRNAs identified, ~ 40% were identified as novel compared to previous published studies, with >700 nucleus-specific [43, 55, 58, 62, 63]. Many of these novel discovered lncRNAs likely result from the different experimental approaches used and/or the gametocyte stages being examined for the first time. In contrast, some of the annotated lncRNAs that were not detected in this study may likely result from the stringent filters used to select intergenic lncRNAs with great confidence. In this study, the stage-specific expression and subcellular localizations of several lncRNA candidates were validated using **RNA fluorescent *in situ* hybridization (RNA-FISH)**. Importantly, the genome-wide occupancy of several lncRNA candidates was also determined by **Chromatin Isolation by RNA Purification (ChIRP-seq)**, a method based on **immunoprecipitation** of biotinylated targeted oligos, and showed that lncRNA occupancy is focal, sequence-specific and correlates with the expression of neighboring genes.

long non-coding RNAs in host cell permeabilization

In the IDC, schizonts express many genes in preparation for the invasion of RBCs, including the merozoite surface proteins and rhoptry-associated proteins [7, 8, 14, 16]. Expression of these invasion-related genes have been shown to be regulated by at least two nuclear proteins: the bromodomain protein PfBDP1, which binds acetylated H3 in promoters, and the transcription factor ApiAP2-I [20, 64]. Suggesting their adaptive roles, some of these genes occur as multicopy CVGs [65, 66], with some regulated epigenetically and expressed variably in parasite laboratory strains and field isolates [23, 67]. For example, the cytoadherence-linked asexual genes (*clag3*), which are involved in forming the plasmodial surface anion channel at the RBC membrane and mediate uptake of soluble host nutrients, include two genes on chromosome 3, *clag3.1* and *clag3.2* [65]. Expression of these genes involves **monoallelic exclusion** mechanism with a memory reset in the mosquito and a nearby lncRNA [38, 65–67]. The epigenetic silencing of *clag3* genes has been linked to resistance against some toxins and antimalarials [38, 67, 68]. Interestingly, both the *clag3* gene switching and biallelic silencing observed in blasticidin S resistance correlate to the

expression of a neighboring, >1kb-long intergenic lncRNA known as TR2, suggesting this lncRNA may activate *clag3* gene switching and/or expression [38] (Figure 3A). However, the function of this lncRNA has not yet been validated at the mechanistic level to elucidate its potential role in epigenetics, monoallelic exclusion, antimalarial resistance and adaptation.

long non-coding RNAs in host immune evasion

After invading the RBC, *P. falciparum* expresses CVGs at the host cell surface. These CVGs, known as **variable surface antigens (VSAs)**, mediate virulence and pathogenicity by two immune evasion strategies that include binding host endothelium (**cytoadherence**) of various tissues to sequester from circulation, and varying the mutually exclusive expression of highly diverse multicopy VSAs (**antigen switching**) [69]. Among VSAs, the ~60 *var* genes have been studied most because they undergo monoallelic expression and encode the main immune evasion VSA – the erythrocyte membrane protein 1 (*Pf*EMP1) [70, 71].

The *var* genes cluster in subtelomeric regions and in five internal chromosome loci [72]. They have different upstream promoter sequences (ups) that form five groups (upsA-E), with one linked to severe malaria (upsA) [73]. Despite those differences, most *var* genes have a similar structure with a highly variable exon 1 and two conserved elements – an intron with a bidirectional promoter and a second exon [74, 75]. The active *var* is expressed strongly in ring stages and downregulated in late-stage parasites when sufficient surface protein is expressed; however, *var* switching occurs in a subpopulation of parasites at a rate specific to each *var* gene [71]. The histone variant H3.3 has been implicated in *var* gene epigenetic memory [76], which is also reset in the mosquito like the *clag*3 genes [11, 67, 77].

Many forms of epigenetic regulation participate in the silencing of inactive *var* genes, including their organization into repressive cluster(s) within peripheral nuclear foci [24, 26–28, 78, 79]. Because Hi-C studies have shown that the 3D chromatin structures of *P. falciparum* strains expressing different *var* genes are highly similar with all *var* genes interacting in a heterochromatin cluster, *var* gene switching likely depends on local factors rather than long-range interactions [27]. At silenced genes, the *var* ups are enriched in repressive marks like H3K9me3, H3K36me3, H2A and HP1 [39, 79–81], as well as the chromatin modifiers histone deacetylase *Pf*Sir2 and methyltransferase *Pf*SET2 (also known as *Pf*SETvs) [82, 83] (Figure 3B). Conversely, the promoter of the active *var* gene is enriched in H3K9ac and the double-variant histones H2A.Z/H2B.Z [32]. While histones in the active *var* gene promoter are also marked by H3K4 di- and tri-methylation, the modifying enzymes responsible are not yet identified [39, 80]. Recently, an immunoprecipitation study has shown a protein complex of the histone acetyltransferase *Pf*MYST and the RuvB-like AAA+ ATPase homologues (*Pf*RuvBL) interacts with the subtelomeric *var* gene promoters and H3, H4, H3K9me1 and H4ac during the ring stage of the IDC, when active *var* gene expression is strongest [84].

Although less is known about which factors mediate local changes in the chromatin states of *var* genes, lncRNAs have been shown to play important roles in their regulation [37, 39–41, 71]. Two lncRNAs are expressed by the *var* intronic bidirectional promoter: a conserved

sense lncRNA extending into exon 2 and a variable antisense lncRNA complementing the 3'-region of exon 1 [74, 75]. These *var*-lncRNAs, which are 5'-capped but not polyadenylated, associate with chromatin and localize to perinuclear foci [75]. Because *var* sense lncRNAs are not specific to individual genes, are transcribed from both active and repressed genes and accumulate in the mature life stages [71], they have been suggested to potentially participate in *var* gene silencing, imprinting or epigenetic memory [37]; however, their functional role has not yet been determined.

In contrast, only the *var* intronic antisense-lncRNA (*var*-as-lncRNA) of the active gene is transcribed by early life stages [71]. Despite the mechanism being ill-defined, *var*-as-lncRNA associates with the active *var* gene in *trans* and initiates its expression in a sequence-specific, dose-dependent manner [39]. Likewise, interrupting *var*-as-lncRNA expression downregulates the active *var* gene and alters its epigenetic imprinting, resulting in *var* gene switching [39, 40]. In contrast, the *var*-as-lncRNA is not necessary for activating the evolutionary divergent *var2csa*, which shows hierarchically selective expression, possibly to mediate a role in gene switching [85]. Therefore, the *var*-as-lncRNAs may have *var* gene- or subtype-specific roles in gene regulation. Recently, RNA-FISH studies on transgenic parasites with controllable *var* gene switching have shown that the *var*-as-lncRNA colocalizes with the active *var* gene in the nuclear periphery [41]. Collectively, these studies demonstrate that the *var*-as-lncRNA regulates *var* monoallelic exclusion in the IDC with roles in gene activation and switching.

In addition to these *var*-lncRNAs, two novel exonic antisense and downstream intergenic groups of *var* lncRNAs have recently been identified [63]. While little is known on their roles, it seems that the regulation of this multi gene family may be mediated by a complex interaction of numerous parasite *var*-lncRNAs.

Beyond the *var*-lncRNAs, other regulatory ncRNAs, lncRNAs and proteins have been implicated in regulating *var* genes and other subtelomeric VSAs. For example, the fifteen RNAs of unknown function (RUF)6 ncRNAs, which are 135 nt long, transcribed by RNA Polymerase III and have >50% GC content, are found in close proximity to the internal *var* genes and expressed in a clonally variant manner [86–88]. These RUF6 ncRNAs colocalize in *trans* with the telomeric and internal *var* ups, and their overexpression can upregulate active *var* gene expression [86, 87]. Similarly, **CRISPR-Cas9** interference of RUF6 expression downregulates the expression of *var* genes and other VSAs like *rifin*, *stevor* and *pfmc-2TM* [88]. In contrast, these GC-rich transcripts were also shown to act as *var* gene repressors in *cis* and suggested to inhibit heterochromatin spreading by acting as insulator elements [86]. Functional knockdowns of two parasite exoribonucleases, *Pf*RNaseII and Rrp6, led to the upregulated expression of RUF6, *var* genes and other VSAs, suggesting their post-transcriptional regulatory roles may be mediated by nascent transcript decay [89, 90]. Furthermore, disrupting *PfRNase*II overcame monoallelic exclusion of *var* genes by increasing transcript abundances of upsA-type *var* and *var*-as-lncRNA, which was further supported by decreased *PfRNase*II expression observed in severe malaria patients [89]. Recently, several ApiAP2 TFs termed heterochromatin-associated factors (ApiAP2-HFs) have been shown to be differentially enriched in heterochromatin, VSAs like *var* genes

and the *ruf6* genomic loci suggesting these TFs may mediate the influence of RUF6-ncRNA on *var* gene expression [20].

Proximal to the *var* genes and other VSAs in subtelomeric regions, lncRNAs transcribed from the *P. falciparum* telomere-associated repetitive elements, or TARE-lncRNAs, could be implicated in forming and maintaining local heterochromatin near these important immune evasion genes [53, 58, 91, 92]. Six repetitive blocks of TARE DNA sequences span from the telomeres (TARE1) to the first subtelomeric *var* gene (TARE6) [91]. Early RNA-FISH studies showed TARE-lncRNAs localize to the nuclear periphery and are transcribed throughout the IDC mainly as >six- and ~four-kb long transcripts spanning TARE6 and from TARE3 to TARE1 [43, 53, 92]. TARE-lncRNAs share similarities with TERRAs, like enrichment with binding sites for the homologues of TERRA-associated nuclear and chromatin-remodeling proteins. The TARE-lncRNAs may regulate telomeric heterochromatin by recruiting chromatin modifiers (i.e., *Pf*Sir2 and *Pf*KMT1) and repressive hallmarks (i.e., *Pf*HP1, *Pf*ORC1 and H3K9me3) [78, 93–95]. For TARE6-lncRNA, these interactions have been suggested to be mediated by secondary hairpin loops formed by 21-bp DNA repeats [92]. Palindromic *var* subtelomeric promoter element 2 (SPE2) motif sequences have also been identified in the TARE-lncRNAs, suggesting they may interact with *var* genes and regulate them by recruiting histone modifiers [53]. Furthermore, ApiAP2-HFs and ApiAP2-SIP2 have also been shown to affect *var* gene regulation and bind SPE2 motifs at TARE regions and upsB-type *var* genes [20, 96], suggesting ApiAP2-complex may interact with these loci or lncRNAs. Supporting this notion, the GC-rich and highly conserved TARE4-lncRNA was recently confirmed to be highly expressed throughout the IDC by RNA-FISH and shown to have strong, specific interactions with the telomeres and regions upstream of the upsB-type *var* genes by ChIRP-seq experiments [43, 53, 58]. While this data will have to be further validated at the experimental and mechanistic level, *P. falciparum* TARE-lncRNAs may be involved in controlling telomere maintenance, heterochromatin clusters and *var* gene expression.

Most recently, nucleic acid secondary structures of guanine tetrads known as G-quadruplexes (G4s), which interact with lncRNAs to influence telomere maintenance, DNA repair, recombination and gene expression in higher eukaryotes, have also been identified in the *P. falciparum* genome [97–100]. Parasite G4s were found to associate with telomeric and subtelomeric regions and to be enriched in VSA genes in all *Plasmodium spp*. [98–100]. Consistent with their potential role in telomere maintenance, the telomere-binding protein, *Pf*GPB2, was shown to interact with DNA G4s and guanine-rich RNA [101]. Parasite G4s were also shown to associate with subtelomeric recombination events [100]. Furthermore, the knockdown of a RecQ helicase, which are known to resolve DNA secondary structures like G4s, has demonstrated higher frequencies of *var* gene recombination, especially for the *var* genes containing G4s [102].

Additionally, G4 ligands have been shown to affect parasite growth, replication and gene expression, particularly in subtelomeric VSAs like *var* genes [100, 103, 104]. Supporting this regulatory role, G4s are enriched in nucleosome depleted regions and the *var* ups, TSSs and exon 1, with an antisense strand preference for the latter [100]. Importantly, an antisense G4 was also shown to repress the ectopic expression of a VSA reporter gene. Strikingly,

a recent study examining multiple CRISPR-Cas9 gene edited parasite knockouts of the RecQ1 helicase resulted in the silencing of all *var* genes [105]. In contrast, RecQ1 genetic knockouts based on a double-crossover showed upregulation of *var* genes in some clones [102]. Although these discrepancies may be due to different approaches, these results should be interpreted cautiously. Regardless, given their associations with *var* gene expression and enrichment proximal to the *var*-as-lncRNA, it is tempting to speculate that lncRNAs and G4s may cooperate to control *var* gene expression.

Although less is known about regulatory proteins that interact with *P. falciparum* lncRNAs, a recent study on transgenic parasites with controllable *var* switching, predicted secondary structures of *var*-as-lncRNAs and subsequently targeted them by **domain-specific ChIRP (dChIRP)** and **LC-MS/MS** to identify their associated proteins [41]. Knockdown of one such protein, the redox sensor thioredoxin peroxidase I (*Pf*TPx-1), demonstrated its role in *var* gene switching and activation at the ring stage, possibly via redox mechanisms providing an ideal microenvironment for transcription [41]. Additionally, *Pf*TPx-1 knockdown showed upregulation of VSAs, like the *var*, *stevor*, *rifin* and *Pfmc-2TM* genes [41]. A related approach, **ChIRP-MS**, identified proteins interacting with the RUF6 ncRNAs [106]. These ncRNAs were found to interact directly with RNA Polymerase II at the *var* expression site as well as a DEAD box RNA helicase, which may potentially regulate gene transcription by resolving G4s of the active *var* gene [106].

long non-coding RNAs in gametocytogenesis

As an alternative to continuing the IDC by reinvading RBCs, a parasite subset can develop into transmissible sexual stages. Parasite commitment to gametocytogenesis is also an adaptive response to the host and ecological factors that affect transmission potential [5, 107, 108]. Our understanding of the transcriptional changes underlying this process and the epigenetic factors regulating them has grown in recent years [109, 110].

The 3D chromatin structure of gametocytes differs substantially from its asexual counterparts [28]. Specifically, the transcription factor *ap2-g*, expressed during the early stages of sexual commitment and essential for gametocytogenesis [5, 109, 110], was shown to interact strongly with repressive heterochromatin in asexual stages but dissociate from them in early gametocytes to reassociate in late-stage gametocytes [28]. The gametocyte development protein 1 gene (*Pfgdv1*) was discovered and shown to be mutated in a gametocyte-deficient laboratory parasite strain [111]. Full restoration of *Pfgdv*1 demonstrated its centrality in regulating gametocytogenesis [111]. During sexual commitment, the nuclear protein *Pf*GDV1 evicts HP1 from H3K9me3 on the *ap2-g* promoter [42]. Following HP1 displacement, euchromatin forms and facilitates the expression of *ap2-g*, which initiates sexual commitment. In the asexual stages, the five-exon antisense lncRNA transcribed downstream from *gdv1* (*gdv1*-as-lncRNA) was shown to self-regulate, repress *gdv1* and indirectly silence *ap2-g* [42] (Figure 3C). Proposed regulatory mechanisms of *gdv1*-as-lncRNA include transcriptional interference of *gdv1* expression, competitive binding of *gdv*1 inhibiting transcription factors and binding of *gdv1* mRNA influencing turnover [37]. Regardless of the exact mechanisms underlying *ap2-g* repression by *gdv1*-as-

lncRNA, regulation of a master ApiAP2 TF by parasite lncRNA suggests other similar regulatory relationships may also exist.

More recently, the male development gene 1 (*pfmd1*) has been demonstrated as critical to parasite sex determination and male gametocyte development via CRISPR-Cas9 genome editing strategies [112]. Using scRNA-seq, this study showed that *md1* expression was male-specific, increased at the sexual bifurcation between gametocyte stages II and II and that a truncated *md1* transcript and an intronic, antisense lncRNA (*md1*-lncRNA) were female-specific [112]. Despite normal expression of *md1* transcripts and protein, sexual maturation and exflagellation, the knockout of the lncRNA-associated intron showed not only a 5-fold lower *md1*-lncRNA expression but also a subtle increase in male gametocytes, suggesting that this lncRNA influences *md1* silencing in female gametocytes but is not the main driver [112]. Overall, this study revealed that sex determination in *P. falciparum* is at least partially regulated by lncRNAs.

With >500 novel candidate lncRNAs recently identified as enriched in the sexual parasite life stages [43], *gdv*1-as-lncRNA and *md1*-lncRNA are likely the first of many regulatory lncRNAs to be found important for *P. falciparum* sexual development. Two of those recently identified candidate lncRNAs lie between genes involved in sexual regulation and were shown previously by **ChIP-seq** for H3K9me3 to be repressed at asexual stages but active at sexual stages [28]. Based on scRNA-seq data, these gametocyte-specific lncRNAs were shown to be gender-specific with higher lncRNA-ch9 and lncRNA-ch14 levels in male and female gametocytes, respectively [43]. Importantly, ChIRP-seq data demonstrated these lncRNAs are enriched near their genomic loci in sexual stages and correlate negatively with H3K9me3 occupancy based on ChIP-seq [28]. Together, these studies suggest these lncRNAs may recruit chromatin remodeling enzymes and sequence-specific TFs to regulate nearby gametocyte-specific genes. To further validate the importance of lncRNA-ch14, disrupting this lncRNA through CRISPR-Cas9 gene editing technology was shown to significantly decrease rates of gametocytogenesis, skew the parasite population sex ratio to male gametocytes and impair male exflagellation [43]. Similarly, membrane feeding assays show mutants were less infectious to mosquitoes based on lower oocyst and sporozoite infection rates and intensities [43]. Collectively, the findings confirm the importance of regulatory lncRNAs to gene regulation throughout *P. falciparum*'s life cycle and highlight a critical need to further understand their role in a more systematic manner.

lncRNAs in host-pathogen interaction

As mentioned previously, circular lncRNAs can interact with miRNAs to alter gene expression. Circular RNAs have been identified in *P. falciparum* and contain potential binding sites for human miRNAs, providing a possible link for crosstalk between parasite lncRNA and host miRNA to influence both parasite and host gene expression [58, 113, 114]. While the plasmodial genomes lack the pathways and molecular components essential for endogenous miRNA biogenesis, it has been well documented that the host miRNAs can be altered by infection and affect the expression of parasite VSA genes [113, 114]. Specifically, these miRNAs have been implicated to affect host cellular immunity, sickle cell protection, susceptibility in pregnancy and the development of severe and cerebral malaria

[113, 114]. Additionally, it has been demonstrated that the parasite can export **extracellular vesicles (EVs)** containing some of these miRNAs complexed with host-derived Argonaute 2 protein, along with parasite mRNA and small non-coding RNAs [113, 114]. These EVs have been shown to affect host vascular permeability, and exogenous EVs added to parasite cultures showed they affect VSA gene expression [113, 114]. Beyond host miRNAs, mouse models have shown malaria infection also affects the expression levels of host lncRNAs implicated in immune-related signaling pathways and host cellular immunity [115]. Overall, these studies suggest that both the parasite and host lncRNAs may have important roles in mediating the host-pathogen immune signaling network to modulate the outcome of the infection.

Concluding remarks

While most of the work regarding the discovery of lncRNAs in *Plasmodium spp*. has been done on laboratory reference strains, it will be interesting to determine the conservation and diversity of lncRNAs among strains and species. Future studies examining lncRNAs in field isolates in varying environmental conditions, such as global regions, antimalarial interventions, malaria endemicities and transmission settings, will be needed to provide a contextual understanding of their potential biological importance to parasite survival and adaptation.

It is also important to keep in mind that RNA-seq approaches, including sc-RNA-seq, often miss lncRNAs using the standard bioinformatic pipelines as these lncRNAs are not always annotated. As lncRNAs are less abundant and stable than steady-state mRNAs [43], it will be critical to improve their detection by using novel strategies together with the depletion of nascent mRNAs, rRNAs and tRNAs [36]. As bioinformatic advances continue to refine the *in silico P. falciparum* lncRNA transcriptome [63], applying these algorithms to datasets enriched for *Plasmodium* lncRNA will allow the detection of additional candidates [43]. However, a new set of experimental approaches is ultimately needed to unravel the role and the biological complexity of parasite lncRNAs [116]. Some of these approaches are described in Figure 4 and include RNA-FISH or CRISPR-Cas9 editing tools that can validate the localization of the identified lncRNA and improve our understanding of their function, respectively [43]. While CRISPR-Cas9 makes site-specific edits with high efficiency, off-target cleavage and disruption of the parasite genome can be a disadvantage. Such an approach can indeed disrupt important DNA loci and DNA binding sites. For the functional analysis of lncRNAs, the adaptation of the **CRISPR-Cas13** system to *P. falciparum* will be a clear advantage. CRISPR-Cas13 is an outlier in the CRISPR world as it can specifically knockdown or edit RNAs, rather than DNAs, in eukaryotic cells. Such a system will allow the functional analysis of parasite lncRNAs without altering the parasite genome sequence [117]. Additionally, lncRNA-knockdown could be used in conjunction with Hi-C, RNA-seq or ribosome profiling to examine their respective contribution(s) to the *P. falciparum* 3D genome, transcriptome and translatome (Figure 4).

Other techniques able to examine the interaction of lncRNAs with DNA, RNAs or proteins are also now widely used to examine the biological function of lncRNAs (Figure 4). As mentioned previously, ChIRP-seq has recently been applied to discover the binding sites

of *P. falciparum* lncRNAs across the genome [43]. Similarly, dChIRP and ChIRP-MS have been used to identify proteins interacting with parasite lncRNAs [41, 106]. However, methods for examining lncRNA-RNA interactions like crosslinking of matched RNAs and deep sequencing (COMRADES) have yet to be adapted to *P. falciparum* [118]. Additional strategies that permit the *de novo* global discovery of the lncRNA interactome with DNA, RNA or protein have the potential to transform the way we look at lncRNA in gene expression and chromatin structure. For example, Chromatin-associated RNA sequencing (ChAR-seq) and RNA *in situ* conformation sequencing (RIC-seq) are based on proximity ligation and could be applied to examine the lncRNA interactome with DNA and RNA, with the latter additionally permitting RNA conformation capture [118] (Figure 4). Also adaptable to *P. falciparum*, the RNA-dependent proteins (R-DeeP) method uses density gradient ultracentrifugation with quantitative MS to identify and estimate the protein complexes that are dependent of RNAs based on RNase-treated and -untreated samples [119]. We are only starting to understand the features and functional versatility of lncRNAs in malaria parasites and it is most likely that this knowledge represents only a small fraction of their regulatory potential (see **Outstanding Questions**). The identification of novel lncRNAs in *Plasmodium spp*. as well as in field samples together with the use of new tools and technologies to understand their function will not only transform the way we see lncRNAs in parasite biology but will also most likely provide novel diagnostic and therapeutic strategies to negatively influence parasite survival in its host cells.

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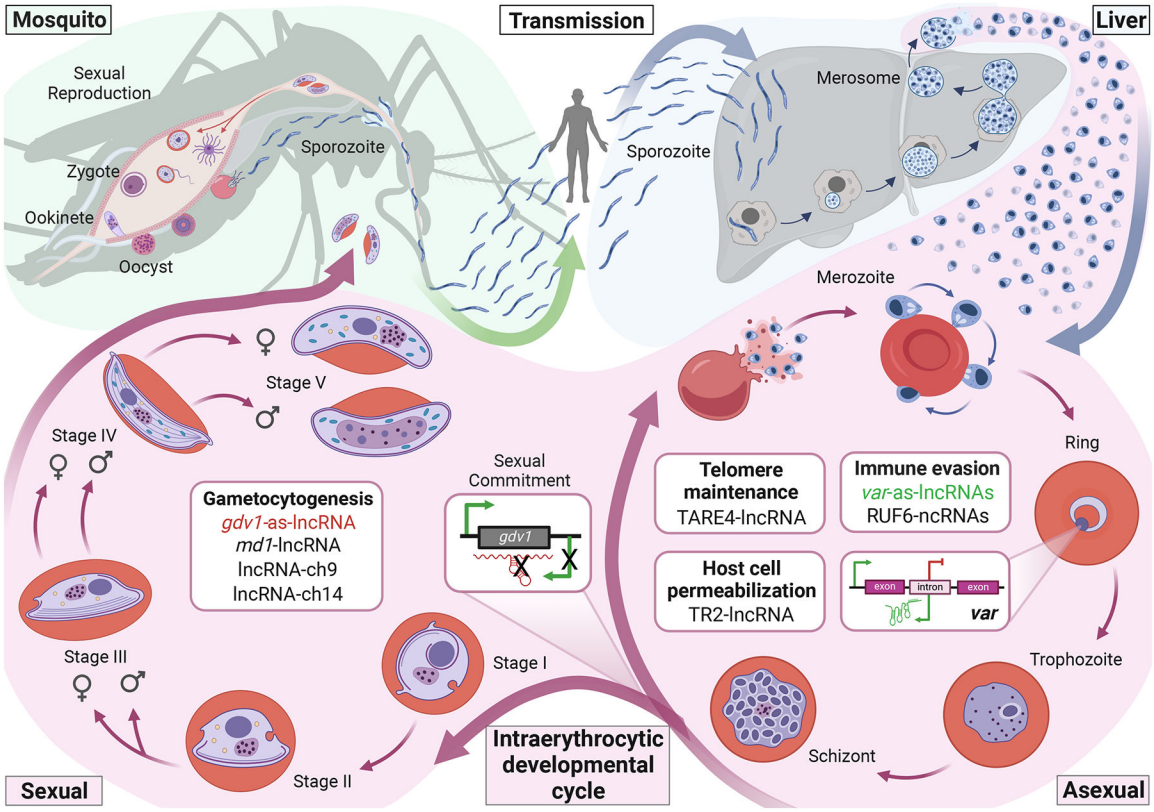

Figure 1. The *Plasmodium falciparum* Life Cycle and key lncRNAs identified.

P. falciparum undergoes many cellular transformations in its hosts leading to distinct morphological and physiological stages. Infection begins when an infected mosquito inoculates sporozoites into the human skin that migrate to the bloodstream. Some of these sporozoites reach the liver to invade hepatocytes where they differentiate and divide into ten to a thousand merozoites within a merosome that reenters the bloodstream. After this 8–14 day process, these merozoites are released to invade erythrocytes and initiate the asexual intraerythrocytic developmental cycle (IDC). Inside anucleated red blood cells (RBCs), parasites develop from relatively dormant ring stages to transcriptionally active trophozoites and then to dividing schizonts that produce up to 32 new merozoites. These merozoites are then released into the bloodstream to invade new RBCs. This asexual cycle lasts approximately 48 hours and can cause symptoms like fever, headache, anemia, multiple organ failure and coma. During the IDC, a portion of parasites may differentiate into male and female gametocytes for transmission. After roughly two weeks of maturation, both sexual forms must be ingested by a female anopheline mosquito. Once inside the mosquito midgut, sexual reproduction leads to the formation of a zygote, which then differentiates into a mobile ookinete and crosses the midgut wall to form an oocyst. The oocyst undergoes schizogony to produce thousands of sporozoites that invade the salivary glands to infect a human host during the mosquito's blood meal, continuing the pathogen's life cycle. Key *P. falciparum* lncRNAs identified to be involved in host cell permeability, immune evasion and gametocytogenesis are listed in their respective stages of the IDC. Additionally, the stage-specific roles of *var*-as-lncRNA in *var* gene activation at the ring stage and *gdv1*-as-lncRNA

in sexual commitment are highlighted (green arrows represent active gene promoters, while those in red are repressed).

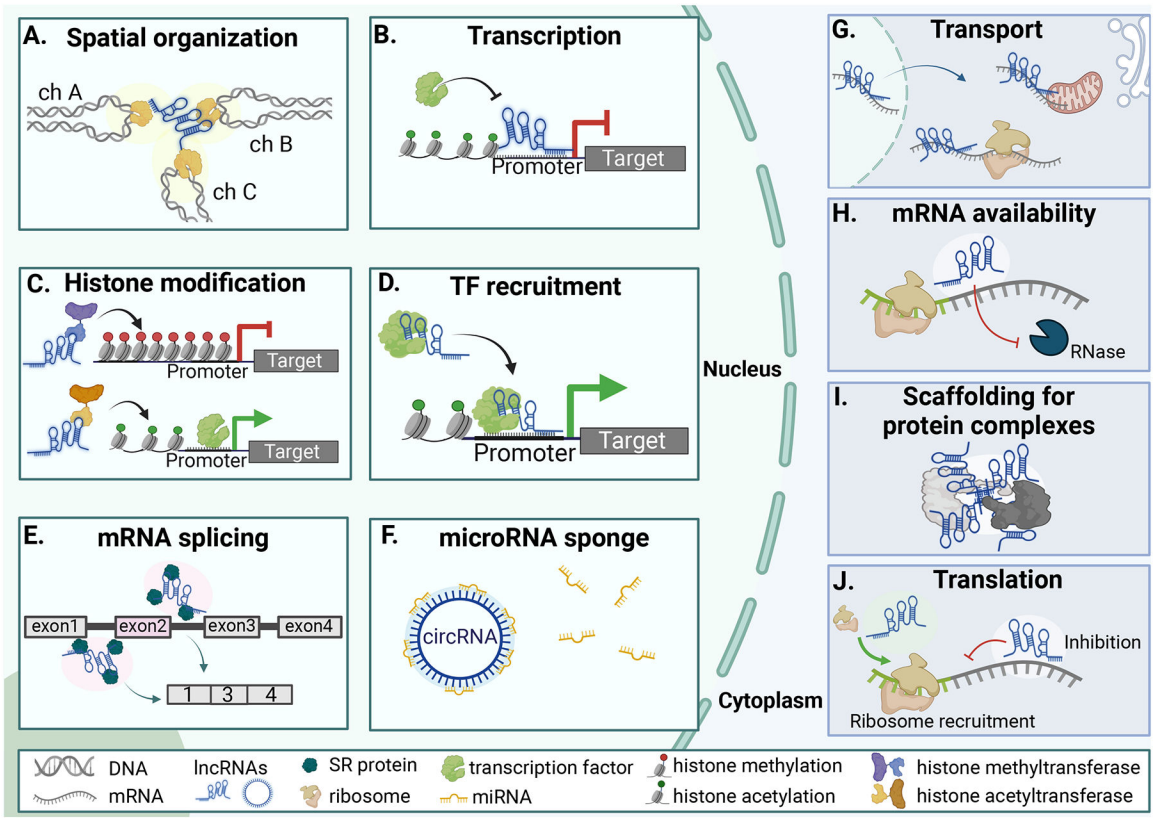

Figure 2. The role of lncRNAs in higher eukaryotes.

Within the nucleus, the functions of lncRNAs in eukaryotic gene regulation include roles in **(A)** spatial organization, **(B)** transcriptional regulation based on DNA binding, **(C)** recruitment of activating and repressing histone modifiers, **(D)** recruitment of transcriptional factors, **(E)** scaffolding for spliceosomal proteins and **(F)** sponging microRNAs. At the post-transcriptional level, lncRNAs can mediate mRNA **(G)** transport and **(H)** availability for turnover, decay or translation. LncRNAs can also **(I)** serve as scaffolds for protein complexes involved in gene regulation and **(J)** interact with translational factors to enhance or inhibit ribosomal translation.

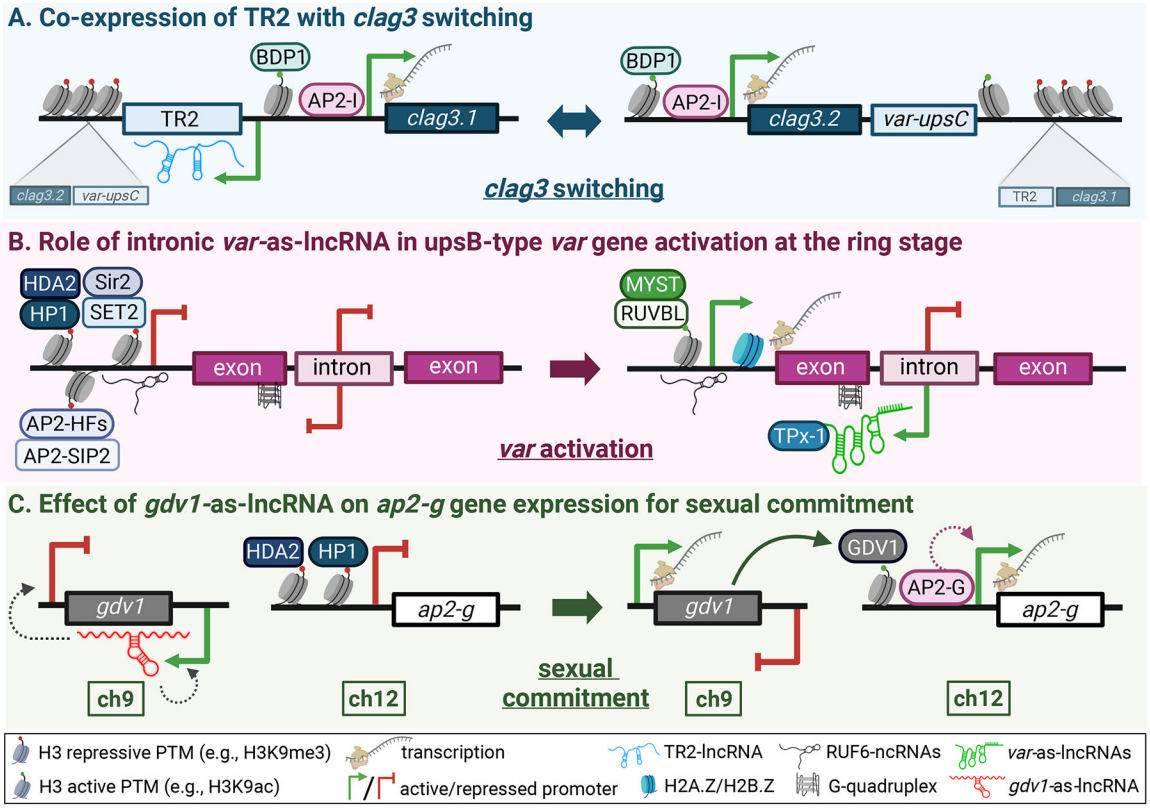

Figure 3. Functional roles of lncRNAs and ncRNAs in *P. falciparum*.

P. falciparum lncRNAs can activate or silence genes relevant to the parasite's IDC and adaptation: **(A)** *clag3* gene switching modulates nutrient uptake, **(B)** *var* gene activation mediates immune evasion and **(C)** *ap2-g* activation initiates sexual commitment. The different shapes used for individual lncRNAs are meant to distinguish them rather than represent their actual structures.

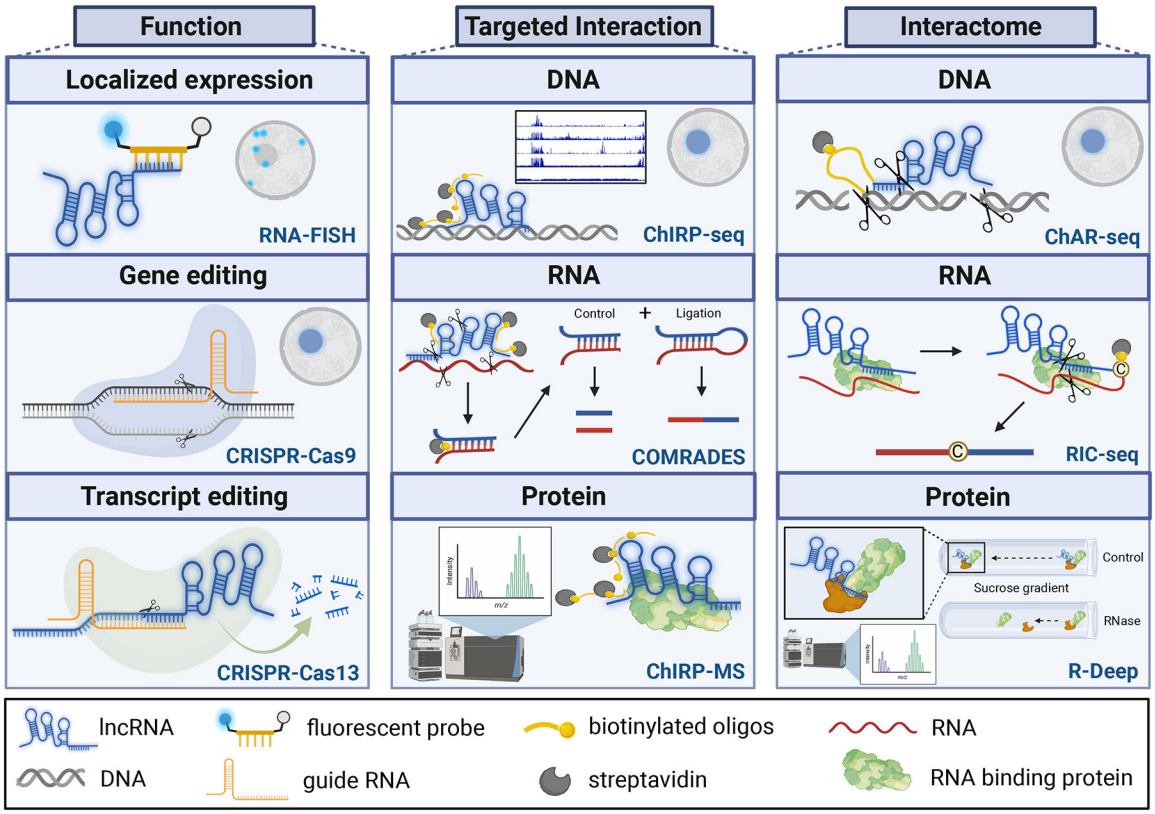

Figure 4. Technological approaches for examining the function of *P. falciparum* lncRNAs.

An array of techniques exists within a systems biology approach for examining the characteristics and relevance of lncRNAs involved in host-parasite interactions. At the DNA, RNA and protein levels, integrating targeted (*a priori*) methods to examine molecule-specific interactions and global (*de novo*) approaches to provide interactome-wide information will help to unravel the complex biology of lncRNAs in malaria. For example, RNA-FISH (fluorescent *in situ* hybridization) can be used to validate the expression and localization of lncRNAs. Genome editing technologies including CRISPR-Cas9 and -Cas13 can be used to knockout or knockdown lncRNA, respectively. Chromatin Isolation by RNA Purification (ChIRP)-seq and crosslinking of matched RNAs and deep sequencing (COMRADES) use biotinylated capture oligos against RNAs of interest to affinity purify crosslinked DNAs and RNAs, respectively. Based on next-generation sequencing, these techniques can identify RNA binding sites. ChIRP-MS uses a similar capture methodology but is followed by mass spectroscopy to identify the proteins that are bound to specific lncRNAs. Global approaches like Chromatin-associated RNA (ChAR)-seq often use proximity ligation followed by affinity purification to identify lncRNA and DNA interactions across the genome. Similarly, RNA *in situ* conformation sequencing (RIC-seq) uses proximity ligation and affinity purification to capture RNA-RNA interactions. Finally, the RNA-dependent proteins (R-DeeP) method uses RNase-treated and -untreated samples coupled with sucrose density gradient, ultracentrifugation and quantitative MS to identify protein complexes that depend on RNAs.