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• Reviews •

Epigenetic regulations in immune evasion of the deadliest malaria parasite *Plasmodium falciparum*

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Abstract Both eukaryotic and prokaryotic pathogens infect the host stably via an immune evasion mechanism termed mutually exclusive expression. Nowadays, little is known about this epigenetic mechanism, largely limiting the understanding of pathogenesis of many bacterial, fungal and protozoan pathogens and therefore the development of novel drugs and vaccines. In the most severe malaria parasite, *Plasmodium falciparum*, there is a major virulence gene family termed *var*, by which the variant antigen PfEMP1 is encoded and expressed on the surface of parasite-infected erythrocytes. Each parasite carries about 60 antigenically various *var* genes, however, only one of which is expressed at a given time during infection. *P. falciparum* expresses PfEMP1s in this clonally variant manner to bind to different human endothelial receptors, allowing the infected erythrocytes to sequester in tissues to escape the host's immune response including spleen killing and humoral immunity. At present, the mechanism of mutually exclusive expression of the *var* gene family remains largely unknown, even though there is increasing evidence suggesting important roles of the epigenetic regulation involved in *var* gene expression. In addition, epigenetic factors were also found in association with transcriptional regulation of other antigenic variant gene families in *P. falciparum*. In this paper, we review the current understanding of epigenetic regulations of *P. falciparum* virulence genes with particular views toward the design of novel vaccines, drugs, and diagnosis to malaria.

Keywords Epigenetics; Histone modifications; Long non-coding RNA; Transcription; Immune evasion; Antigenic variation; Malaria; *Plasmodium falciparum*

1 Introduction

Malaria is a major parasitic disease in humans [1]. Human malaria could be caused by five different species among the unicellular protozoan parasites of the *Plasmodium* genus, of which the most deadly form *Plasmodium falciparum* causes 300—500 million clinical cases and more than 1 million deaths worldwide each year [2]. In Africa alone, malaria kills a child (mainly count on children under the age of 5 infected by *P. falciparum*) in every 45 seconds due to the lack of effective malaria vaccines or novel antimalarial drugs [3]. The effectiveness of current therapeutic strategies is attenuated by increasing resistances of malaria parasites to the clinically used drugs [4]. The most ideal chemotherapy plan recommended by the WHO is Artemisinin Combination Therapy (ACT), which is currently the frontline therapy for the asexual blood stage of the malaria infection [5]. However, the parasite appears to be developing resistance to artemisinin-based drugs, the most effective antimalarial currently in use [6]. The emerging resistance to

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artemisinin in some areas of Southeast Asia is now a looming threat to the global public health [4]. On the other hand, no effective malaria vaccines have been available yet since the most potential vaccine candidate RTS, S/AS01 was proven at a poor protection level [7—10]. At present, the development of innovative vaccines with high protection efficacy and screening of chemical compounds as potential novel antimalarial drugs is urgently needed as a top world health priority. Obviously, success of these purposes would benefit from the knowledge of regulation mechanisms of key virulence genes in *P. falciparum* [11, 12].

Both eukaryotic and prokaryotic pathogens can infect the host stably via an immune evasion mechanism termed mutually exclusive expression [11]. It has been known for decades that *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is a critical virulence factor for malaria, playing important roles in immune evasion mechanism to protect parasites from the host antibody response [12, 13]. PfEMP1 binds human endothelial receptors allowing the parasitized erythrocytes to sequester in tissues and keep the parasite from passing through the spleen where they are killed by macrophages [13, 14]. PfEMP1 is also responsible for the two major severe complications of malaria: cerebral malaria by binding to brain endothelium and metabolic acidosis by interfering with blood flow to muscle and other organs [15—17]. The *P. falciparum* genome contains about 60 antigenically distinct *var* genes that are expressed clonally by each parasitized erythrocyte [18—21]. Antibodies elicited to PfEMP1 expressed by an infecting parasite can block sequestration resulting in an increase of parasite killing in the spleen [13]. Immune elimination of the infecting parasite allows the expansion of parasites that have switched on the expression of a new *var* gene to which the elicited antibodies have no protective effect [12, 16]. Thus, the education of the immune system to recognize all *var* gene products is a slow process that can take years of exposure under malaria infection [13]. The molecular basis of mutually exclusive expression of *var* genes is poorly understood [12]. Nowadays, mounting evidence suggests that various epigenetic factors including histone modifications and long non-coding RNA (lncRNA) account for the molecular regulation of *var* genes [22—29]. Furthermore, histone modifications have also been indicated to play important roles in other *P. falciparum* antigenic gene families, such as erythrocyte binding-like proteins (EBL), reticulocyte binding protein homologues (RH) and clag families [30—33]. Here, we highlight the recent progress in understanding the epigenetic regulation of parasite genes with a particular view towards *var* gene expression and postulate how these findings may benefit or lead to successful controls of malaria.

2 Summary of epigenetic regulations of *P. falciparum* antigenic gene families

The life cycle of *P. falciparum* comprises a sexual stage taking place in mosquito, a pre-erythrocyte stage in liver cells, and an asexual blood stage in erythrocytes when the symptoms of the disease appear [1, 34]. There are two most significant events of parasite growth during the asexual blood stage. One is parasite invasion to erythrocytes mediated by the parasite ligand including EBL or RH proteins and their host receptors on the surface of the infected erythrocytes [6, 34]. The other is sequestration of infected erythrocytes via PfEMP1 proteins binding to endothelial receptors in the microvasculature of different organs [1, 6]. Parasites could evade human immune responses in epigenetic manners either by using alternative invasion pathways using EBL and RH proteins [1, 35] or by switching expression of *var* genes to avoid spleen killing [11, 13, 27]. It has been demonstrated that no DNA methylation [36] or RNAi mechanism [37] occurs in *P. falciparum*, suggesting histone modifications and lncRNA may contribute to transcription regulation of *P. falciparum* genes [38, 39]. Global histone analysis by mass spectrometry indicated that methylation and acetylation were also enriched at the common lysine site of histone proteins except H3K27 [40, 41]. Interestingly, either sense or antisense lncRNAs are also dominated in the parasite transcriptome, suggesting its important roles in gene regulation in spite of the absence of RNAi in *P. falciparum* [42].

RH5, a member of RH family, is a critical virulence factor in a sailic acid-dependent invasion pathway by *P. falciparum* into human erythrocytes [43]. Our laboratory has found that trimethylation at H3K9 (H3K9me3) inactivated the expression of the *Rh5* gene as an epigenetic memory marker throughout the a-

sexual stage [30]. In contrast, H3K4me3 is related to the activation of *Rh5*, in association with the acetylation of H3K4 and H3K9 (H3K4ac and H3K9ac) [28]. Moreover, nucleosome density around the transcription start site (TSS) of *Rh5* is also related to its activation [35]. The similar regulation by histone acetylation and H3K4/9me3 in expression of other members of EBL and clag gene families has also been characterized by other research groups [33]. Besides *var* genes, other antigenic gene families such as *rifin* and *stevor* can also encode variant surface antigens (VSAs) that were also suggested to contribute to immune evasion, even though the biological functions of the resulting proteins RIFIN and STEVOR still remain unknown [27]. Our recent work suggested that H3K36me3 may play a role in silencing of these genes, in association with histone deacetylation at H3K4 and H3K9 controlled by at least one of *P. falciparum* histone deacetylases (PfHDACs) (Figure 1) [39]. At present, transcriptional regulation of *var* genes is most well-studied due to the important role of PfEMP1 proteins in pathogenesis of *P. falciparum*. We therefore mainly focus on this area in this review as discussed below.

3 Epigenetic factors for *var* gene expression

Many protozoan parasites including *P. falciparum* express VSAs in a clonally variant manner to escape the host's immune response [11]. It has been assumed that antibodies to VSAs encoded by gene families such as *var*, *rifin* and *stevor* may contribute to protective immune responses [27]. In *Giardia lamblia*, only one out of more than 200 VSA genes is expressed during the life cycle of a given parasite clone [44]. Knockdown of RNA-dependent RNA polymerase or Dicer genes has been shown to activate the simultaneous expression of multiple VSAs [44] and appears promising for the development of a whole parasite vaccine [45]. Mutually exclusive expression of *P. falcipaurm* VSA genes is regulated at the transcriptional level by histone deacetylation and H3K9me3 in the 5′-UTR of the genes [22, 25]. However, expression of the majority of the *var* gene family members is not activated by disruption of two HDACs (PfSir2A/B) [46], indicating *P. falciparum* histone lysine methyltransferases (PfHKMTs) and histone lysine demethylase (PfHKDMs) or other unknown transcriptional regulators, such as lncRNA and *var*-specific transcription factors, may play more important roles in silencing of VSA genes.

3.1 Histone modifications

Interestingly, *P. falciparum* displays unique genomic characteristics such as the absence of linker histone H1 [47], the absence of RNA interference (RNAi) machinery [37], the presence of DNA cytosine methyltransferase but apparent absence of DNA methylation [36, 48], and the presence of unusual histone variants with a distinct set of modifications [41]. Mutually exclusive expression of *var* genes has been first reported to be associated with two PfHDACs called PfSir2a and PfSir2b, however, neither of which is the key silencing factor for all the *var* genes [23, 24]. As in other eukaryotic cells, the histone lysine methylation controls the expression of *var* genes in a finer manner than histone acetylation does in *P. falciparum* [49].

In eukaryotic cells, there are a large number of proteins functioning as histone modification enzymes (such as methyltransferases, demethylases, acetyltransferases, and deacetylases), and others containing the domains that participate in the recognition of specific histone modifications (chromo, bromo, PHD, tudor domains, etc.) [50—54]. Crosstalk between these two types of epigenetic factors decides the fate of gene expression. Histone lysine methylation is involved in both transcriptional activation and silencing and is regulated by the opposing actions of PfHKMTs and PfHKDMs in correlation with PfHDACs and acetyltransferases (PfHATs). Bioinformatic analysis indicated that there are totally 10 PfHKMTs belonging to the SET [the Drosophila suppressor of variegation-Su(var), the Polycomb-group protein Enhancer of zeste-E(z) and Trithorax (TRX) group proteins] domain-containing protein family and 5 PfHKDMs, controlling the histone lysine methylation (Table 1) [39, 55]. For histone acetylation, there are totally 5 annotated PfHDACs and 3 Pf acetylases [39]. Some of these enzymes and the corresponding histone modifications have been demonstrated in regulating *var* gene expression (Figure 1) [23, 24, 39, 46]. At present, it has been demonstrated that there are at least 44 different posttranslational modifications in *P. falciparum* histone proteins, some of which are the key factors in controlling *var* gene expression [22, 25, 52].

Table 1 Annotated *PfHKMT* and *PfHKDM* genes in *P. falciparum*

	Name	Gene ID	Inferred activity	Comments
SET domain proteins (Histone lysine Methyltransferases)	PfSET1	PF3D7_0629700	H3K4	Ortholog of Drosophila Trx/Human Mll
	PfSETvs	PF3D7_1322100	H3K36	Ortholog of Drosophila ASH1, the only representative of the SETD2-NSD-ASH1 clade in *P. falciparum*, a distinctive domain architecture displaying the cysteine-rich AWS domain N-terminal to the catalytic SET domain
	PfSET3	PF3D7_0827800	H3K9	Ortholog of Su(var3H); laterally transferred from plant-lineage
	PfSET4	PF3D7_0910000	H3K36/other chromatin proteins	SMYD clade
	PfSET5	PF3D7_1214200	Unknown	Transferred from a nucleo-cytoplasmic large DNA virus or a bacterial endosymbiont. Viral versions methylate H3K27, but there is no evidence that it catalyzes this modification in *P. falciparum*
	PfSET6	PF3D7_1355300	H3K36/other chromatin proteins	Ortholog of SMYD2
	PfSET7	PF3D7_1115200	H3K36/other chromatin proteins	SMYD clade
	PfSET8	PF3D7_0403900	H4K20	Ortholog of SETD8
	PfSET9	PF3D7_0508100	H3K4	SMYD clade
	PfSET10	PF3D7_1221000	H3K36	An apicomplexan-specific clade believed to methylate H3K4. However, it is not closely related to the conventional H3K4 methylases and is close to the SETD8 clade. It contains a N-terminal PHD finger and C-terminal tudor domain that was not previously identified
LSD1	PfLSD1	PF3D7_1211600	H3K4me1 and H3K4me2	LSD1 family; has PHD finger at C-terminus unlike animal/plant LSD1s
	PfLSD2	PF3D7_0801900	H3K4me1 and H3K4me2	LSD1 family; has PHD finger at C-terminus unlike animal/plant LSD1s
Jumonji	PfJmjC1	PF3D7_0809900	Histone lysine demethylase	Member of clade prototyped by human KDM5A
	PfJmjC2	PF3D7_0602800	tRNA wybutosine hydroxylase	Ortholog of tRNA wybutosine hydroxylase from other eukaryotes
	PfJmj3	PF3D7_1122200	Probably a protein N-hydroxylase	JMJ4 clade

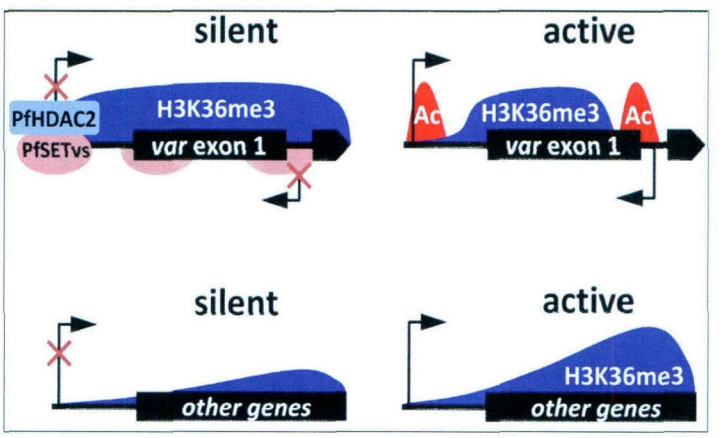

Figure 1 The diagram shows that the PfSETvs-dependent H3K36me3 enriched along the entire gene body of silent *var* genes including the TSS of *var* genes and the respective intronic antisense promoter leads to silencing of both *var* mRNA and antisense lncRNA. PfHDAC2 may contribute to deacetylation of Histone H3 at the TSS of silent *var* genes.

3.1.1 Histone H3 lysine methylations

Histone lysine methylation in the mutually exclusive expression of *P. falciparum var* genes has been studied intensively since this phenomenon was discovered. H3K4me2/3 but not H3K4me1 was found at the *var* promoter, in association with active or poised transcription throughout the entire asexual stage [25, 56]. During the roughly 48-hour-cycle of the parasite asexual stage, a single *var* gene is transcribed in the first 24 hours after invasion [21]. It becomes silent during the late asexual stage (25-48 hour after the invasion); however, the resulting translation product PfEMP1 is expressed and presented on the surface of the infected erythrocytes, by which parasites can bind to various endothelial receptors such as CD36, intercellular adhesion molecule 1 (ICAM1), platelet/endothelial cell adhesion molecule (PECAM), CR1, heparin sulfate and chondroitin sulfate A (CSA) [57]. The di-and trimethylation at histone H3K4 were the further suggested function in the promoter region as the epigenetic memory marker for re-activation of the *var* gene at the onset of the next cycle of the asexual blood stage [25]. However, the enzyme catalyzing H3K4me2/3 specifically for *var* gene activation remains unknown [56]. This obviously restricts the exploration of the molecular mechanism controlling the expression of a single *var* gene. By contrast, histone H3K9me3, a heterochromatin marker, has been shown to be associated with *var* gene silencing [20, 22]. In addition, this modification was also found in a negative correlation with expression of other parasite genes [30, 33]. However, none of these histone modifications was found in controlling silencing of the majority of the *var* gene family [39].

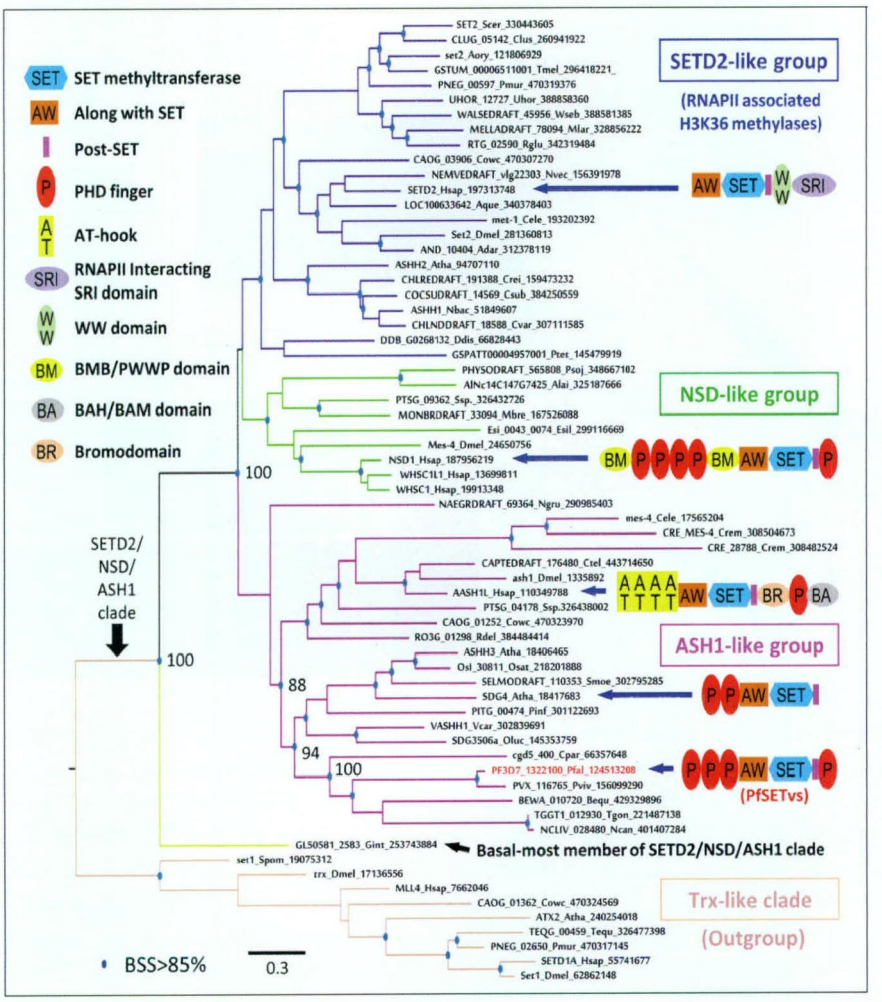

Figure 2 A maximum-likelihood phylogenetic tree of the SET domains of the SETD2-NSD-ASH1 clade rooted using the Trx/Mll-like clade as an outgroup. Select architectures for particular proteins are shown and the actual bootstrap values are shown for the ASH1-like subclade. All clades with BSS>85% are indicated with a circle. The domain abbreviations are shown to the left. The three distinct subclades of the SETD2-NSD-ASH1 clade, namely the SETD2, NSD-like and ASH1-like subclades are shown in distinct colors. The *Giardia intestinalis* member groups outside these three subclades indicate that their divergence happened after the separation of *Giardia* and related excavates from the rest of the eukaryotes.

To further corroborate the role of histone lysine methylations in *var* gene silencing, our laboratory has tried to knock out each of the PfHKMT and PfHKDM genes [49]. The only orthologue of SET2 (PfSETvs) in *P. falciparum* (Figure 2) was therefore identified as a key silencing factor of 59 out of 60 *var* genes [58]. When PfSETvs was deleted, almost all 60 of the *var* genes were transcribed simultaneously in a single parasite (Figure 3(a)) [39]. Moreover, multiple PfEMP1s were also identified on the surface of erythrocytes infected by the PfSETvs KO parasite (Figure 3(b)—(d)) [39]. Global histone modification analysis indicated that PfSETvs may deposit trimethylation on the histone H3K36 along the TSS region of *var* genes for gene silencing [39]. In the same study, other histone modifications such as H3K4me3/H3K9me3/H4K20me3 were shown less correlated with *var* gene silencing [39]. In other eukaryotes, H3K36me3 was known as a common epigenetic marker enriched in the 3′ terminal of actively transcribed genes [59, 60]. For the first time, the work of PfSETvs demonstrated a novel function of H3K36me3 in gene silencing [39]. Considering the ability of H3K36me3 to recruit HDACs to the 3′ region of active genes to avoid the cryptic transcription initiated inside the active genes, the molecular mechanism of H3K36me3 in repressing transcription may also occur in certain inactive genes in other eukaryotic cells (Figure 1). This discovery may explain the correlation of H3K36me3 and unusually silent genes in *Caenorhabditis elegans*, *Drosophila melanogaster*, and zebrafish, and the association between H3K36me3 and pericentromeric heterochromatin in mouse embryonic sgtem cells and fibroblasts [61—64].

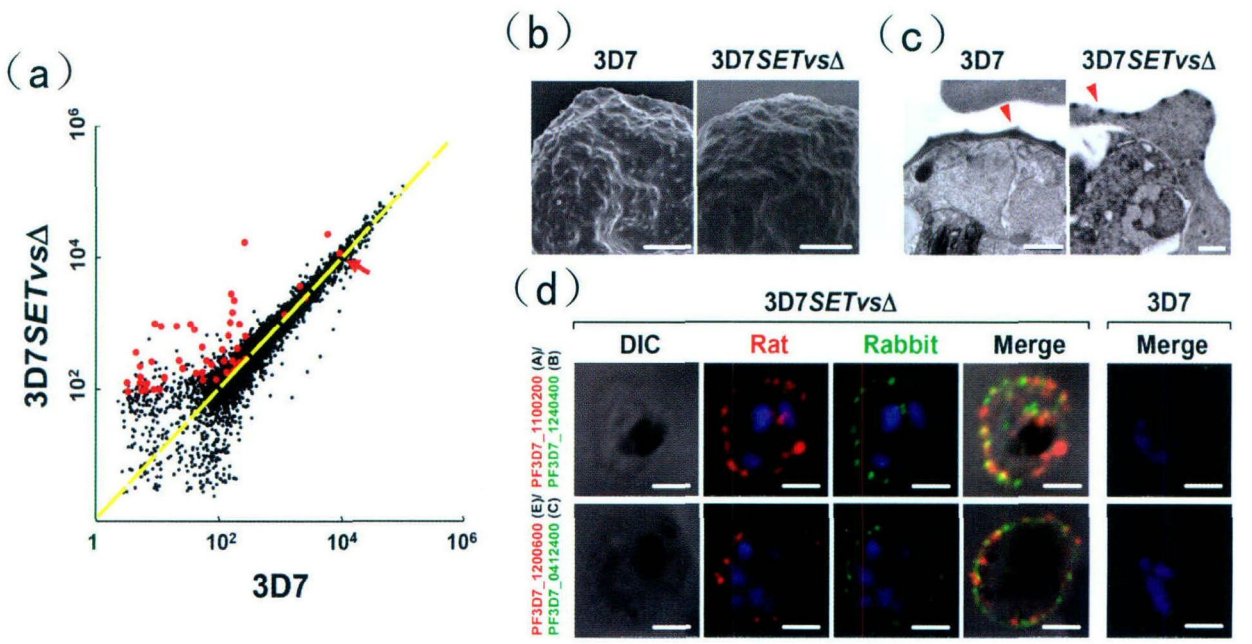

Figure 3 Simultaneous expression of multiple *var* genes in single 3D7*SETvs*Δ iRBCs. (a) Comparative transcriptome analysis of wild-type 3D7 and 3D7*SETvs*Δ at 18 h after invasion. The *x* axis (wild-type 3D7) and the *y* axis (3D7*SETvs*Δ) are logarithmic and correspond to the relative signal of hybridization to each gene shown as a dot. All *var* genes with authentic hybridization signals are shown in red. The dominantly expressed *var* gene (PF3D7_1240600) in wild-type 3D7 is indicated by a red arrow. (b), (c), Electron microscopy of gelatin selected 3D7 and 3D7*SETvs*Δ iRBCs. Typical knobs in scanning electron microscopy (SEM) (b) and transmission electron microscopy (TEM) (c) pictures are indicted by red arrowheads. (d), Live-cell immunofluorescence assay (IFA) using rat and rabbit antisera to various PfEMP1s to detect the co-expression of different PfEMP1s on the surface of 3D7*SETvs*Δ iRBCs. Wild-type 3D7 infected RBC is shown to the right. No staining is seen. DAPI (blue) is used to mark the parasite nucleus. Types of *var* genes are shown in parentheses. Scale bars: (b) 1 μm; (c) 0.5 μm; (d) 1.5 μm.

In addition, genome-wide analysis of the distribution of H3K36me3 indicated that this epigenetic mark was deposited almost exclusively in telomereand subtelomere regions of all the 14 chromosomes in *P. falciparum* (Figure 4(a)) [39]. Interestingly, it was also associated with other antigenic gene families such

as *rifin* and *stevor*. Disruption of PfSETvs also caused coexpression of multiple genes of the two families (Figure 4(b)) [39]. Given the critical role of PfSETvs and the corresponding H3K36me3 in silencing of these important antigenic genes, further investigations on this epigenetic factor controlling immune evasion of *P. falciparum* would be worth pursuing. On the other hand, the key factor controlling the active transcription of *var* genes still remains unknown. Success of such studies will greatly improve understanding this key step of antigenic variation by *P. falciparum*, and therefore benefit the application of exploring malaria vaccines.

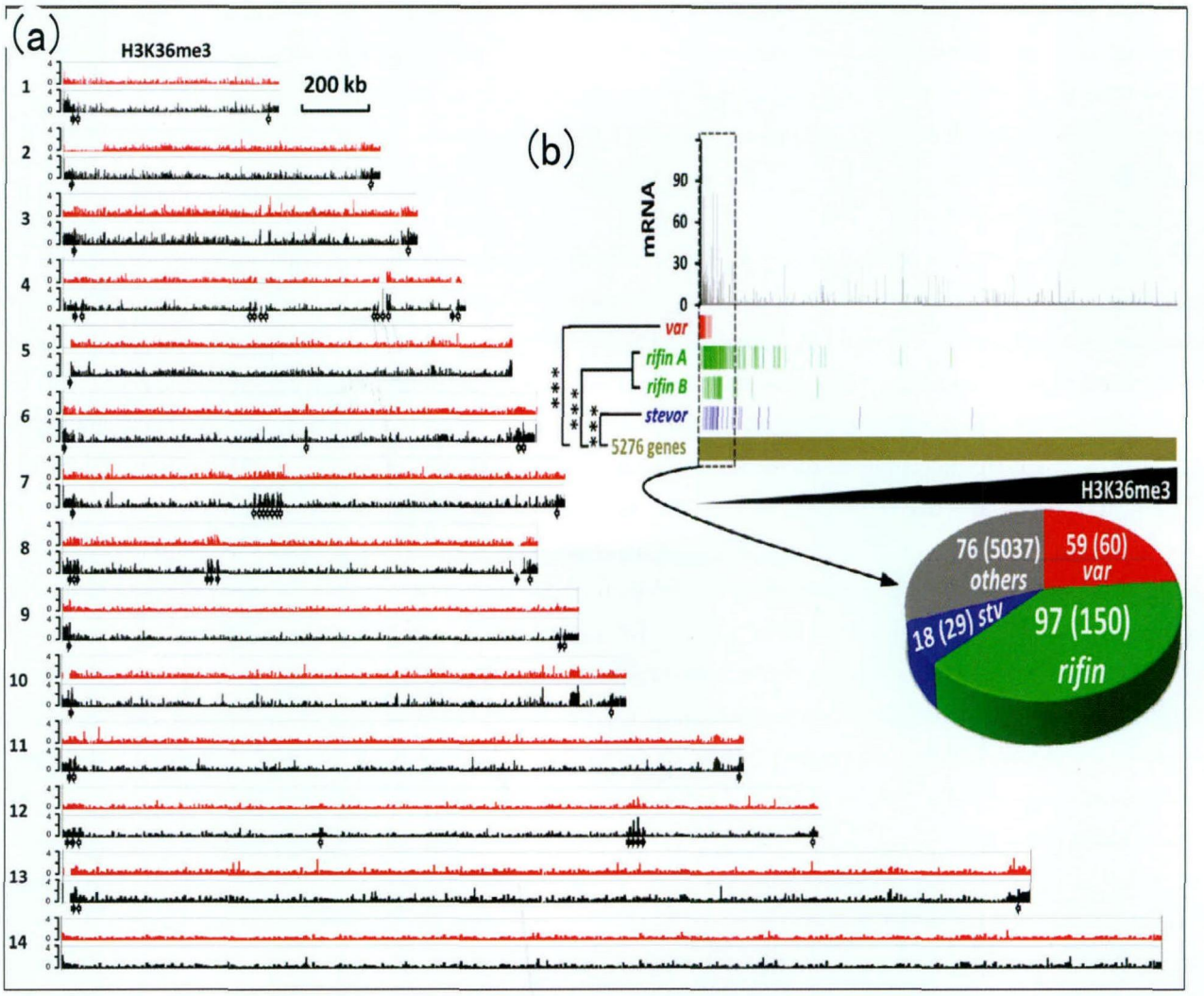

Figure 4 PfSETvs-dependent H3K36me3 is specifically associated with *var* gene silencing. (a) Integrative genomic view of ChIP-seq analysis of H3K36me3 along 3D7 (black) and 3D7*SETvs*Δ (red) chromosomes at 18 h after invasion. Sixty *var* genes distributed along *P. falciparum* chromosomes 1—13 are indicated by solid (forward orientation) and open (reverse orientation) arrows. Each read was normalized by the total number of uniquely mapped ChIP-seq reads. Chromosomal numbers are shown to the left. (b) Statistical analysis of the correlation between reduction of H3K36me3 and upregulation of *var*, *rifin* and *stevor* gene families. 5276 parasite genes were sorted from low to high levels of H3K36me3 in 3D7*SETvs*Δ normalized by that in 3D7. Expression fold change of each gene by *PfSETvs*Δ was shown on the top panel. Distribution of all of *var* (red), *rifin* including A-and B-type *rifin* genes (green) and *stevor* (blue) genes is shown along the parasite genes (gold). In the top 250 H3K36me3-reduced genes boxed by dash lines, numbers of *var* (red), *rifin* (green), *stevor* (blue) and other genes (grey) compared to their total numbers are shown in a pie chart at the bottom. Hypergeometric test was computed for the *var* (P = 3.4e−80), *rifin* (P = 9.7e−98) and *stevor* (P = 1.73e-17) gene families to gauge their significance of upregulation in the reduction of H3K36me3.

3.1.2 Histone H3 Acetylation

PfSir2a was the first identified PfHDAC in silencing of *var* genes by two independent research groups [23, 24]. Implicated by these findings, PfSir2b was further found in regulation of *var* silencing in the sim-

ilar manner as PfSir2a does [46]. Genome-wide distribution analysis of pan acetylation at lysines 4/9/14 of histone H3 indicated that histone H3 acetylation was related to the active transcription of *var* genes [26, 27]. However, disruption of each of the two PfHDAC genes could not activate the expression of all *var* genes, suggesting that none of the PfSir2 proteins accounts for silencing of all of *var* genes [46]. Considering the silencing role of H3K36me3 in *var* genes and its potential interaction with HDACs, at least one of other PfHDACs may broadly silent *var* genes, in association with H3K36me3 in the TSS region (Figure 1) [39]. Recently, Duraisingh's group in Harvard University has claimed that PfHDAC2 would be functional as this kind of key factor. In this work, they have shown that the conditional knockout of the PfHDAC2 gene in parasites resulted in simultaneous expression of all *var* genes (unpublished data), the same phenotype as observed in the study of PfSETvs knockout. Further study to investigate the interaction between PfSETvs and PfHDAC2 will shed light on a better understanding of the epigenetic mechanism of H3K36me3 in gene silencing.

3.2 lncRNA

Non-coding RNAs (ncRNAs) are the RNAs that are transcribed from DNA but are not translated into proteins and play an important role in a variety of processes, including genetic imprinting [65], RNAi-mediated transcriptional repression [66], dosage compensation [67, 68] and involvement in chromatin assembly in eukaryotes, including organisms as varied as dinoflagellates [69], yeast, fruit flies, mice, and humans [70]. Each *var* gene carries at least three different promoters: is the gene promoter for transcription of mRNA, the intronic promoter for sense lncRNA, and the intronic promoter for antisense lncRNA [39]. Transcription of the sense lncRNA has been identified for years in both active and silent *var* genes at the late asexual blood stage of the parasite [71]. This sense lncRNA was suggested as a *var* gene marker for recognition of mutually exclusive expression, while its molecular function in transcription regulation of the *var* gene family remains largely unknown [72]. Recently Deitsch's group in Cornell University discovered a new antisense lncRNA transcribed from the intron region [73]. They further supposed that these lncRNAs might recruit chromatin-modifying enzymes to specific chromosomal regions, thereby influencing genome organization or gene expression [74]. In mice, convincing evidence showed that one of the two X chromosomes in females was expressed during development, involving two lncRNAs, Xist and its antisense transcript Tsix [75—77]. Xist RNA is expressed at a low level in both females and males before differentiation [78]. Upon cell differentiation, Xist RNA coats the future inactive X chromosome (Xi) triggering extensive histone lysine methylation [79], whereas Tsix appears to restrict Xist activity on the future active X chromosome. We demonstrated a correlated upregulation of *var* genes and their corresponding antisense lncRNAs in association with low occupancy of the PfSETvs-dependent H3K36me3 at the TSS (Figure 1) [39]. Although the current evidence implicates a role of the antisense lncRNA in activating *var* genes, the molecular mechanism of this lncRNA is not clear nowadays. The actively transcribed antisense lncRNA may function as a trans-acting element in silencing of other *var* genes, or coactivate its corresponding *var* gene *in cis* by sharing one set of transcription machinery with the *var* promoter [74]. Further study on the spacial interaction of *var* genes at a global level may provide more information about the function of the *var* lncRNA.

3.3 RNA Polymerase II

In other eukaryotes, RNA polymerase II recruits SET2 through the phosphorylated form of its C-terminal domain (CTD) alongside the mRNA elongation in transcriptionally activated genes. A recent report showed that only unphosphorylated CTD of RNA polymerase II binds to PfSETvs, an orthologue of SET2 in *P. falciparum* [74]. Considering the important role of PfSETvs in *var* gene silencing, opposite phosphorylation states in the CTD of RNA polymerase II may contribute to the regulation of *var* genes. It has been known that both silent and active *var* genes could transcribe sense lncRNAs via the intronic promot-

er, which was driven by RNA Polymerase II [38, 71, 72]. Given a potential role of the *var* sense lncRNA as a genetic marker of the mutually exclusive expression of *var* genes [38], RNA polymerase II with an unphosphorylated CTD could block the transcription of the *var* antisense lncRNA by recruiting PfSETvs to its corresponding promoter [39, 74]. For the single active *var*, both the gene promoter and the antisense intronic promoter may utilize RNA polymerase II with a phosphorylated CTD to avoid incorporation of histone H3 with K36 trimethylated by PfSETvs [74]. It will be interesting to investigate whether sense and antisense promoters in the *var* intron recruit RNA polymerase II depending on the phosphorylation state of its CTD. The correlated regulation involving both *var* lncRNA and RNA polymerase II on *var* gene expression needs to be investigated further. This would be greatly helpful to discovering the whole mechanism of *var* gene expression.

3.4 APiAP2 transcription factors

Since the entire genome of the first *P. falciparum* clone 3D7 was fully sequenced in 2002, around 40% of 5500 predicted Pf genes are still functionally unknown [18—20]. Particularly to transcription factors, only an Apicomplexan AP2 (ApiAP2) protein family that contains putative AP2 DNA-binding domains has been widely studied. The *P. falciparum* ApiAP2 (PfAP2) gene family contains 27 members that are conserved across Plasmodium species with almost identical AP2 DNA-binding domains in orthologues from different species with only one exception [80, 81]. These PfAP2 transcription factors were found expressed in different stages of the entire life cycle of *P. falciparum* [81], strongly suggesting the important roles of this protein family in regulation of various Pf genes, and therefore in many events of parasite pathogenesis. In a comprehensive study, the global DNA-binding specificities for each PfAP2 protein was characterized by utilizing a well-designed DNA array [82]. The overall studies on PfAP2 protein family suggested that these proteins are the main components of transcription factors with its binding ability to distinct DNA sequences along promoter regions of different genes in *P. falciparum* [83, 84]. In addition, one of the PfAP2 proteins, named PFSIP2, could function as a scaffolding and recruitment protein in remodeling of Pf chromosome ends, in association with a heterochromatin protein [85]. This opens up a new epigenetic function for this protein family. Some of PfAP2 were also suggested in association with *var* gene transcription. However, little is known to the molecular mechanisms of the PfAP2 proteins due to the large size of these proteins, which limited attempts of overexpressing them in full length into parasites to corroborate the biological function of these transcription factors. Along with the intensive development of gene disruption techniques in *P. falciparum*, it is now possible to knock out more than one target gene in a single parasite clone. Knockout of both PfAP2 and histone modification enzymes that were proven related to *var* gene expression such as PfSETvs would accelerate the current knowledge of mutually exclusive expression of the *var* gene family. Equally importantly, identifying the biological role of other unknown domains that widely exist in this large protein family would also uncover the novel function of these transcription factors. For example, how and when PfAP2 proteins are targeted to the nucleus. Further studies on this critical transcription factor family may also provide molecular evidence for developing these proteins as potential antimalarial drug targets.

4 Conclusion and future prospects

4.1 Design efficient malaria vaccines

Antigens that induce inhibitory antibodies and human immune responses are recognized as the primary candidate for malarial vaccines that target parasite blood stages. The *P. falciparum* apical membrane antigen-1 (AMA1) has been considered as a promising antigen for the development of the blood stage vaccine

[86], however, inhibitory activity and AMA1-induced protection in animal models and humans are highly strain-dependent [87]. In addition, the most promising vaccine candidate RTS, S/AS01 was recently failed in its clinical phase III trials [7—10]. Therefore, development of an effective vaccine is still urgently needed to reduce mortality and morbidity of infants and young children living in malaria epidemic areas. A contemporary view of PfEMP1 is that antibodies to PfEMP1s dramatically reduce malaria infection [13]. Recently, it was shown that PfEMP1 is a key target for humoral immunity [88]. Obviously, discovery of the regulation mechanism for *var* genes in *P. falciparum* will provide a powerful platform for the development of malaria vaccines. For instance, the PfSETvs-knockout parasite that expresses all PfEMP1s on the surface of infected erythrocytes could be utilized as a potential vaccine candidate [89]. By presenting all PfEMP1s at the same time to the human immune system, it would be easier to receive fully protective immunity against the whole PfEMP1 pool [45, 89]. This immunization could greatly relieve patients away from the pain of multiple malaria infection before raising full resistance to the parasite.

4.2 Develop novel antimalarial drugs

Nowadays, many chemotherapy strategies have been built in the malaria treatment. However, it is inevitable that chemotherapeutic drugs could induce drug resistance occurring in certain parasite strains after the long-term use. For example, the chloroquine resistance strains increased mortality and spurred the rush to find an alternative chemical compound. In addition, the most effective antimalarial drug, artemisinin, has also appeared to be resistant in some *P. falciparum* strains in Southeast Asia [4]. The emergence of artemisinin resistance has therefore motivated researchers to identify different molecular mechanisms controlling critical events in parasite pathogenesis for the discovery of new drug targets [90]. It is highly accepted that the high-throughput screening of large chemical compound libraries will produce the next generation of antimalarial drugs with great potential [91]. By utilizing this powerful technique, many institutes and pharmaceutical companies developed a large number of chemical compounds with high ability to kill malaria parasites [92]. At present, most of the compounds were developed based on the Single-Nucleotide Polymorphism (SNP) or copy number variation occurring in target genes and expressed in the asexual blood stage of parasites [92]. However, none of these compounds was targeted to parasite molecules with a known function, partially explaining why there is currently no successful compound in clinic trials [92]. By now, the investigation of immune evasion in *P. falciparum* has greatly expanded our understanding of developing novel antimalarial drugs. Identification of some key enzymes such as PfSETvs related to immune evasion of parasites has provided more targets for compound screening [89]. Moreover, the further study on transcription factors controlling critical pathogenetic events may also contribute to the discovery of new drug targets. For this purpose, it is worth pursuing the role of the PfAP2 protein family in regulating the expression of the key antigenic gene family such as *var* [89].

4.3 Explore accurate diagnostic biomarkers

Malaria causes million clinical cases particularly in pregnant women and children in the tropical areas that are highly populated but less developed [2]. Lacking the efficient vaccine and the raising drug resistance prevents the process towards the eradication of malaria. On the other hand, poverty and backward economical conditions also greatly limit people receiving malaria diagnosis in good time. Nowadays, the most accurate and authoritative diagnosis of malaria is still microscopic examination of blood, even though there are several rapid diagnostic testing (RDT) kits commercially available [93]. Thus, identification of specific and sensitive biomarkers for malaria is still needed nowadays. Metabolomic analysis during the pre-blood liver stage and the asexual blood stage would be a way to provide direct evidence for this purpose [5]. Besides, the discovery of novel posttranslational modifications in non-histone proteins would also benefit the characterization of new malaria biomarkers.

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