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Variant Gene Expression and Antigenic Variation by Malaria Parasites

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Abstract

Malaria is a significant threat throughout the developing world. Among the most fascinating aspects of the protozoan parasites responsible for this disease are the methods they employ to avoid the immune system and perpetuate chronic infections. Key among these is antigenic variation: By systematically altering antigens that are displayed to the host's immune system, the parasite renders the adaptive immune response ineffective. For *Plasmodium falciparum*, the species responsible for the most severe form of human malaria, this process involves a complicated molecular mechanism that results in continuously changing patterns of variant-antigen-encoding gene expression. Although many features of this process remain obscure, significant progress has been made in recent years to decipher various molecular aspects of the regulatory cascade that causes chronic infection.

Contents

PLASMODIUM FALCIPARUM AND THE PATHOGENESIS OF MALARIA

Despite extensive effort to reduce the burden of malaria in the last decade, it remains one of the most devastating infectious diseases on Earth. Recent advances notwithstanding, the World Health Organization's goals of reducing global malaria burden by 40% by 2020 and 90% by 2030 are unlikely to be met (20). This potentially fatal disease is caused by protozoan parasites of the genus *Plasmodium*, which are transmitted to humans through the bite of female anopheline mosquitoes. *Plasmodium* parasites have evolved a complex life cycle, alternating between hosts and environments with specific molecular adaptations at each stage, including both sexual and asexual phases. Although all stages are important for completion of the parasite's life cycle as well as for transmission to the mosquito vector, only the blood stage causes disease. More than 200 *Plasmodium* species are known to infect a variety of vertebrates, but only 5 cause malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Together, *P. falciparum* and *P. vivax* are responsible for approximately 90% of malaria cases. The vast majority of mortality, however, is caused by *P. falciparum*, which is therefore considered the causative agent of the deadliest form of the disease (reviewed in 21).

All *Plasmodium* species invade and replicate within their hosts' erythrocytes, but the specific pathogenicity of *P. falciparum* is attributed in part to its ability to modify the surface of human infected red blood cells. These modifications impart cytoadhesive properties to the red blood cells, enabling them to sequester and obstruct small blood vessels and leading to damage in deep tissues, including the lungs, kidneys, and placenta. In addition, sequestration within the brain can cause life-threatening pathologies in a syndrome known as cerebral malaria (98). Cytoadherence and sequestration evolved to enable *P. falciparum* to avoid passage through the spleen; thus, this process successfully allows the parasites to escape filtration and mechanical clearance from circulation. However, because of their exposure to the extracellular environment, surface molecules responsible for the cytoadhesive properties of infected red blood cells are also the primary antigens exposed to the immune system, which readily generates a strong antibody response. To evade this arm of the host's adaptive immunity, the parasites have evolved unique mechanisms of tightly controlled alternating expression of adhesive surface protein variants (27). This process of antigenic variation is summarized in **Figure 1**.

PfEMP1, *var* GENES, AND ANTIGENIC VARIATION

The variable cytoadhesive surface antigens are collectively known as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) and are encoded by a multiple-copy gene family named

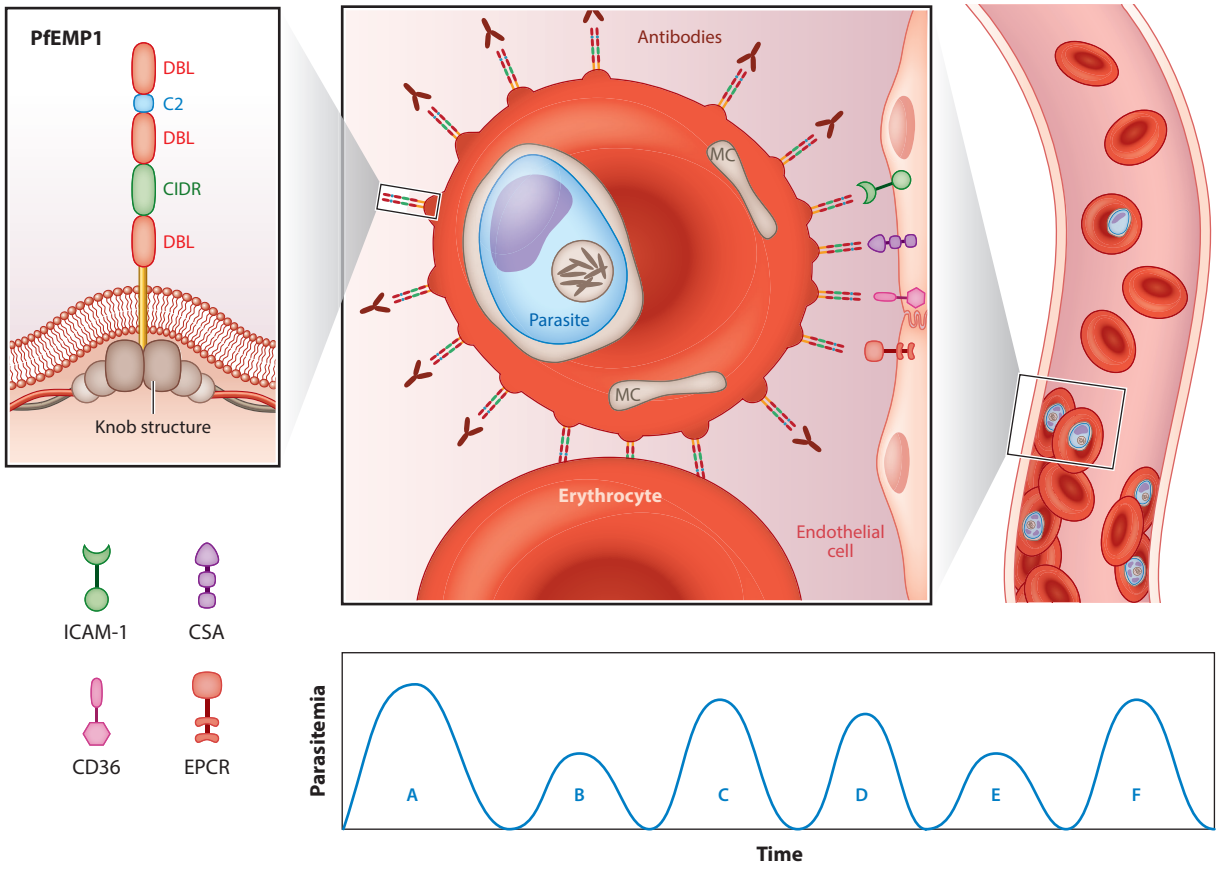

Figure 1

Cytoadhesion and antigenic variation by *Plasmodium falciparum* malaria parasites. An infected red blood cell (*middle inset*) displays PfEMP1 on its surface, anchored into membrane distortions referred to as knobs (*left inset*). The extracellular portion of PfEMP1 consists of a series of domains called DBL (Duffy binding-like), CIDR (cysteine rich interdomain region), and C2 (86) that bind to receptors found on the surface of endothelial cells, including CD36 (cluster determinant 36), CSA (chondroitin sulfate A), ICAM-1 (intercellular adhesion molecule 1), and EPCR (endothelial protein C receptor). Infected cells can also adhere to uninfected red blood cells. Adherence to the vascular endothelium results in sequestration away from the circulation and obstruction of blood vessels (*right*). Antibodies arise that recognize specific forms of PfEMP1, disrupting adhesion and leading to destruction of large portions of the parasite population. Populations of parasites that have switched to expressing alternative forms of PfEMP1 can then expand, resulting in sequential waves of parasitemia over time (*bottom*). Abbreviation: MC, Maurer's cleft.

var (8, 85, 87). Each parasite has approximately 60 *var* genes in its genome, with each gene encoding a different variant of PfEMP1, but only one *var* gene is expressed at a time—a phenomenon known as mutually exclusive expression. Over time an antibody-mediated response targeting the expressed form of PfEMP1 develops and can profoundly reduce the population of parasites in circulation, oftentimes to such low parasitemias that infected cells cannot be detected by microscopic examination of stained blood smears (60). However, small numbers of parasites within the infecting population can switch expression to a *var* gene that encodes a PfEMP1 variant that has not yet been exposed to the immune system. This switch allows the parasites to once again undergo clonal expansion until they trigger another antibody-mediated response. Thus, switches in

Mutually exclusive expression: tightly regulated expression of multicopy gene families such that only one member is expressed at a time

Epigenetic memory: the tendency for the transcriptional state of a gene to be transmitted through multiple cycles of cellular replication

var gene expression are believed to allow the parasites to evade immunity and maintain chronic infections characterized by oscillating waves of parasitemia (61). To avoid premature exposure of its antigenic repertoire, the parasites tightly maintain mutually exclusive expression so that only a single *var* gene (and the encoded PfEMP1) is expressed at a time, while the remainder of the gene family remains transcriptionally silent (30, 83, 95). In addition, *var* expression switching has evolved to occur at a low rate; thus, exposing the entire antigenic repertoire requires many generations, which can extend a single infection for more than a year. Interestingly, several studies have shown that *var* gene switching does not display a predetermined, hardwired pattern but rather seems to follow a loose hierarchy (5, 45, 76) where each gene turns on and off at its own intrinsic switching rate (37, 67).

Although *P. falciparum* generally maintains a repertoire of approximately 60 *var* genes, this number varies somewhat between field isolates (52). All 60 *var* genes share a similar structure. The PfEMP1-coding region has two exons separated by a conserved intron. Each gene has two defined promoters: The first is upstream of the coding region with the transcription start site located approximately 1 kb upstream of the open reading frame (24, 87). This promoter drives expression of the PfEMP1-encoding mRNA and is subject to mutually exclusive expression. A second bidirectional promoter is found within each *var* intron and gives rise to sense and antisense long noncoding RNAs (lncRNAs) (**Figure 2**) (35). In its sense orientation the intronic promoter appears to be simultaneously active at most or all *var* genes.

Mutually exclusive expression ensures that only a single nascent and steady-state *var* mRNA transcript can be detected in each parasite at a time (30, 50, 83, 95). Over the last decade it has become clear that the mechanisms that regulate mutually exclusive *var* gene expression are complex, involving several layers of regulation. First, *cis*-acting DNA elements and RNA transcripts found at each gene have been implicated in silencing and activation, as well as mutually exclusive expression. Second, transcriptional activation and silencing are each tightly associated with the presence or absence of specific histone marks, thereby implicating various epigenetic regulators and histone-modifying enzymes in determining the active or silent state of a gene. These epigenetic marks were also implicated in maintaining the transcriptional status of a gene through multiple cycles of asexual replication, a concept called epigenetic memory. In addition, there is evidence for the involvement of subnuclear organization in controlling *var* gene expression. Lastly, expression of each gene must be coordinated with that of the other members of the family to ensure that activation of one gene coincides with silencing of the previously active gene, thus maintaining mutually exclusive expression. An understanding of this last layer of regulation remains particularly elusive.

In trying to understand such a complex regulatory pathway, it can be helpful to look at it as the interplay between mechanisms that regulate gene silencing and activation, epigenetic memory, mutually exclusive expression, and coordination of expression switching. Here we review recent work on these topics and synthesize this knowledge in a way that might stimulate ideas for experimental investigations.

HISTONE MODIFICATIONS AND THE EPIGENOME

The epigenome of eukaryotes is defined by specific modifications, typically either posttranslational modifications of histone proteins or DNA methylation, which mark chromatin as either condensed, transcriptionally silent heterochromatin or more open euchromatin that facilitates transcription. Low levels of DNA methylation have been observed in malaria parasites (72), but the significance for gene regulation has not been established. In contrast, the distribution of specific histone modifications, known as the histone code, has been convincingly associated with

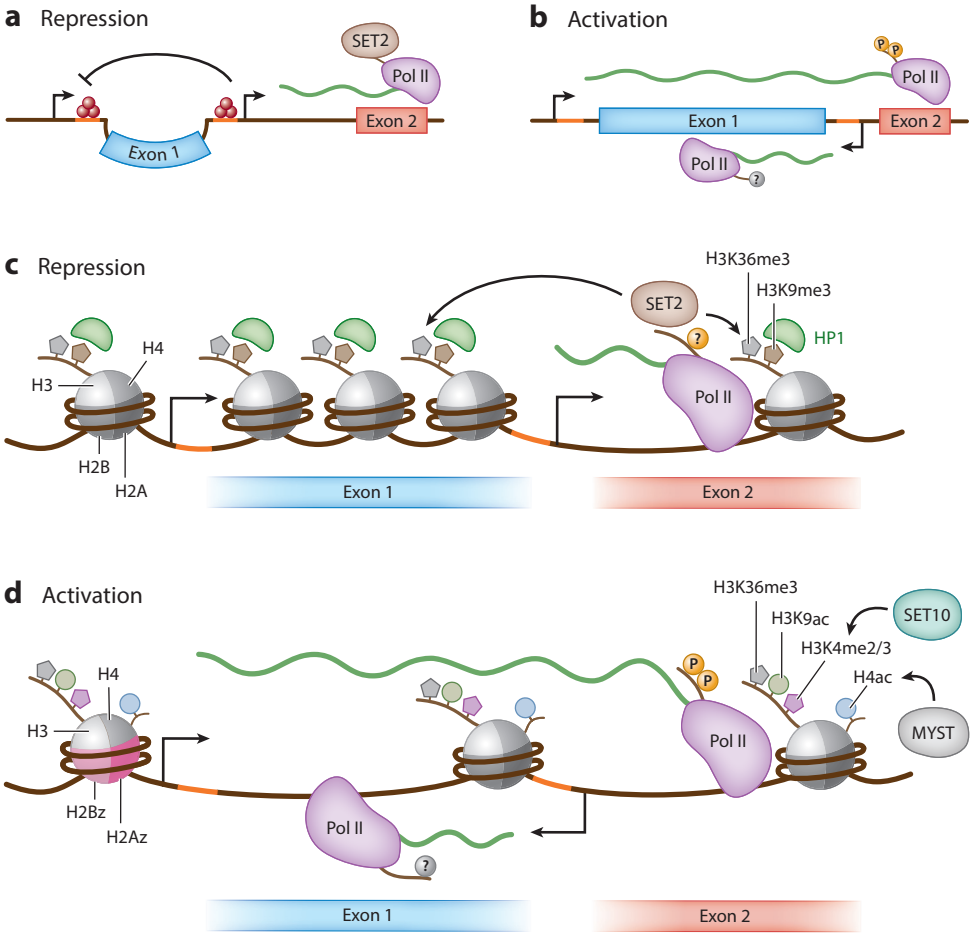

Figure 2

var gene structure and elements of epigenetic transcriptional regulation. (*a*) The two-exon structure of each *var* gene is shown. In the repressed state, transcription by RNA polymerase II initiates from a promoter located within the intron, recruiting the histone modifier PfSET2 to the locus. Two pairing elements (*orange*) are bound by nuclear proteins (*red spheres*) and are proposed to facilitate interactions between the promoters, resulting in silencing of the upstream promoter. (*b*) In the active state, RNA polymerase II transcribes both an mRNA in the sense direction from the upstream promoter and a lncRNA in the antisense direction. The mRNA-transcribing polymerase has a phosphorylated C-terminal domain (CTD), whereas the status of the CTD of the antisense-transcribing polymerase is unknown. (*c*) Schematic representation of a silent *var* gene showing nucleosomes consisting of the histones H2A, H2B, H3, and H4 (*gray spheres composed of four subunits*). The posttranslational modifications H3K9me3 and H3K36me3 are also shown. Recruitment of PfSET2 through binding to the CTD of RNA polymerase II is shown, along with the binding of heterochromatin protein 1 (HP1) to the modified nucleosomes, resulting in condensed, transcriptionally inactive heterochromatin. (*d*) Schematic representation of an active *var* gene showing active transcription by RNA polymerase II of both mRNA and antisense lncRNA. Nucleosomes near the upstream promoter incorporate the alternative histones H2Bz (*light pink*) and H3Az (*dark pink*) and display the posttranslational modifications H3K9ac, H3K4me2/3, H3K36me3, and H4ac. The histone modifiers PfSET10 and PfMYST are recruited to the locus to transfer the modifications H3K4me2/3 and H4ac, respectively, although the mechanisms of recruitment are unknown. These histone modifications and the action of RNA polymerase II are proposed to perpetuate a more open chromatin structure. Abbreviations: Pol II, RNA polymerase II; SET, Su(var)3-9, Enhancer-of-zeste and Trithorax; MYST, moz, Ybf2/Sas2, Sas2, and Tip60.

epigenetic gene regulation in *Plasmodium*. Several studies have characterized the genomic landscape of histone modifications in *Plasmodium* species using various methods based on chromatin immunoprecipitation [ChIP, ChIP-on-chip, and more recently ChIP-seq (36, 43, 56, 80, 100)]. Although there are some differences between their conclusions, a consensus has emerged that *P. falciparum* has a unique epigenome when compared to model eukaryotes (80). In particular, two histone marks typically found distributed throughout the genomes of higher eukaryotes, triple methylation of the 9th and 36th lysines of histone H3 (H3K9me3 and H3K36me3), appear to be specifically devoted to gene families that undergo clonally variant transcription, including *var* genes. Work from several groups has clearly demonstrated that deposition of these specific histone marks at *var* genes influences their expression (18, 36, 55). Specifically, the nucleosomes assembled within the chromatin at the promoter regions of silent genes display H3K9me3, whereas the active gene displays an acetylation mark at this same position (H3K9ac). In contrast, H3K36me3 has been associated with both active and silent *var* genes, although its distribution over the length of the gene changes with the gene's transcriptional state (47, 91). Regions marked by H3K9me3 are then bound by heterochromatin protein 1 (PfHP1) leading to condensation of the chromatin fiber and silencing of transcription (36, 69). The importance of these marks for proper *var* gene regulation was confirmed by experiments in which expression of the enzymes responsible for depositing the marks (the histone methyl transferase PfSET2 or histone deacetylase PfHda2) or PfHP1 was disrupted (10, 19, 47). These led to complete disruption of *var* gene regulation, resulting in expression of the entire family simultaneously. Similar studies in which orthologues of the histone deacetylase SIR2 (PfSir2a and PfSir2b) were knocked out were initially interpreted as demonstrating that these enzymes play a role in *var* gene regulation (28, 90), although more recent work has cast some doubt on these conclusions (58).

In addition to changes in histone modifications, the promoter regions of active and silent *var* genes are also associated with specific nucleosome variants. The promoter region of the active *var* gene contains histones with specific variants of H2A named PfH2A.Z and PfH2B.Z. These "double variant" nucleosomes are enriched with the euchromatic marks H3K9ac and H3K4me3 and are localized to key regulatory regions in the genome (7, 70, 71). PfH2A.Z and PfH2B.Z have been proposed to have reduced stability and therefore more easily dissociate from the DNA, thereby allowing more efficient transcriptional activation (44). Two histone-modifying enzymes have been implicated in the deposition of histone modifications that mark active *var* genes, the acetyltransferase PfMYST that acetylates histone H4 and the methyltransferase PfSET10 that methylates H3K4 (59, 94). Interestingly, PfSET10 localizes exclusively to the active *var* gene in late stages of the asexual cycle when this *var* gene is not undergoing transcription but rather is poised for transcription in the next cycle. This suggests that PfSET10 plays a role in transmission of epigenetic information during parasite replication and division and contributes to heritable epigenetic memory (94). A summary of the epigenetic modifications that are thought to influence *var* gene regulation is shown in **Figure 2**.

NUCLEAR ARCHITECTURE AND SUBNUCLEAR LOCALIZATION

Dynamics of nuclear architecture and the distribution of the chromosomes within the nucleus have been linked to changes in the epigenome and have also been postulated to play a role in *var* gene regulation. Using Fluorescent In Situ Hybridization (FISH) it was initially observed that silent *var* genes cluster into 6–8 foci at the nuclear periphery, colocalizing with so-called "telomere bouquets" (39). The active *var* gene, while similarly localized within the periphery, appeared to be excluded from these clusters, leading to the suggestion that the active *var* gene "moves away" from heterochromatic nuclear domains and into a euchromatic region that allows transcription

(28, 75). The presence of a *var* specific expression site was supported by subsequent studies employing transgenic lines containing episomal *var* promoters that were impaired in silencing and therefore constitutively active. These promoters colocalized to the same subnuclear position as the active, endogenous *var* gene (31, 95). These studies, together with the specific colocalization of PfSET10 with the active *var* gene, support the hypothesis that *var* gene localization is dynamic and correlates with transcriptional activation or silencing. However, the episome studies indicate that this site can accommodate more than one active *var* promoter at a time (29, 31, 95), showing that while it is important for *var* gene activation, it appears not to contribute to mutually exclusive expression. Dynamic changes in *var* positioning at the nuclear periphery were suggested to be mediated by interactions between nuclear actin and a sequence element present in *var* introns (102), a potentially novel mechanism of nuclear organization.

WHAT KEEPS A VAR GENE OFF?

The ~60 *var* genes that reside within the genome of any given parasite are typically found either within the subtelomeric regions of most chromosomes or arranged as tandem arrays within internal chromosomal regions (40). Simple sequence analysis of the coding and upstream regulatory regions indicates that there are five basic *var* gene types referred to as A-E (49, 53). Types A and B are found in multiple copies and are generally located within the subtelomeric regions, while type C genes are more commonly found within the internal regions of the chromosomes. Type D (also called *var1csa*) is a single copy, conserved pseudogene of unknown function and type E is the highly conserved gene also known as *var2csa*. Expression of this gene has been linked to pregnancy-associated malaria due to the binding of the encoded PfEMP1 to chondroitin sulfate A on the syncytiotrophoblasts of the placenta (78, 79). Regardless of the *var* gene type, it appears that all but a single member are maintained in a transcriptionally silent state. Therefore understanding what keeps a *var* gene silent is important for understanding the basic mechanisms underlying antigenic variation.

Early experiments employing plasmids and transfected parasite lines were used to identify specific DNA elements found near *var* genes that play a role in transcriptional regulation. Initial studies indicated that removal of a *var* upstream region/promoter from its chromosomal context caused it to become transcriptionally active and unrecognized by the mechanism controlling mutually exclusive expression (23). However, "pairing" the *var* promoter with a *var* intron caused it to revert to the silent state in an S-phase dependent manner. Subsequent work further indicated that the promoter activity of the intron was required for this effect, hinting at a possible role for RNA polymerase II or the resulting noncoding RNAs (lncRNAs) (14). Interestingly, these lncRNAs are localized within the nucleus, nonpolyadenylated and chromatin associated, but their function is unknown (35). More detailed dissection of DNA elements found upstream of type C *var* genes identified a short sequence element called MEE that appears to contribute to mutually exclusive expression (11), although the mechanism by which this element contributes to controlling expression remains unexplored.

Additional experiments utilizing reporter gene constructs explored the interactions between *var* upstream regions and the DNA elements found within *var* introns. These studies indicated that a *var* intron could only silence a single *var* upstream promoter at a time, further leading to the hypothesis that these two regions "paired" in some way (38, 88). In addition, short DNA elements are found in both regions that are bound by nuclear protein complexes and mediate the pairing phenomenon (3) (**Figure 2*a***). Importantly, these "pairing elements" reflected the strict pairing requirement previously observed, i.e., deviation from the 1:1 ratio by deletion of one pairing element or addition of extra elements disrupts the interactions between a *var* upstream

promoter and intron, resulting in constitutive activation of the gene and loss of mutually exclusive expression (3). The sequence similarity of the two elements and the fact that they compete with one another for binding to a distinct nuclear complex in electrophoretic mobility shift assays suggest that they are potentially interchangeable and could physically interact, serving as anchors for a hypothetical chromatin loop that could form at each gene.

WHAT TURNS A VAR GENE ON?

As described above, all but a single member of the *var* gene family are silenced at any given time through mechanisms that maintain *var* upstream promoters transcriptionally inactive. These mechanisms result in the imprinting of these loci with specific epigenetic marks associated with silent heterochromatin (18, 55). Although recent studies advanced our understanding of silencing mechanisms and the maintenance of heterochromatin, very little is known about the mechanisms involved in the activation of the individual *var* gene that transcribes a PfEMP1 encoding mRNA. Considering the similar structure of all *var* genes and their high degree of sequence similarity, any mechanism of activation must be specific enough to differentiate one *var* gene from another to enable gene-specific "choice" for activation. Moreover, many chromosomal regions contain tandemly arrayed clusters of *var* genes in which the genes are separated by only a few kb, yet one can be transcriptionally active while the neighboring genes are silent. This pattern indicates that each gene is regulated individually rather than through the modification of large chromosomal domains, and that the regulatory mechanism can separate adjacent active and silent genes within the same locus. However, to date, specific boundary elements that separate the heterochromatic environment of silent *var* loci and the euchromatic niche of the active gene have not been identified. In addition, while several protein-binding DNA elements were identified within *var* 5′ regulatory regions (15), no specific transcription factor has been implicated in *var* gene activation.

There is one potential regulatory element that does contain gene specific characteristics: antisense lncRNAs transcribed from the intron of the single, actively expressed *var* gene (**Figure 2*b***) (1, 35, 47). Unlike the sense lncRNAs transcribed from the introns of all members of the family, these antisense lncRNAs can be detected only from the active *var* gene during the early stage of the asexual cycle when the gene is also actively transcribing mRNA, but not later in the cycle when the active gene is poised for transcription (1, 47). These transcripts are approximately 2 kb long and include two regions: a conserved sequence near their 5′ end that includes the highly conserved intron sequence and a gene-specific sequence within the 3′-end that includes a highly polymorphic region of *var* exon 1 (**Figure 2*b***). These antisense transcripts are incorporated into chromatin and there is evidence that they are involved in *var* gene activation. Specifically, a silent *var* gene could be activated by expressing its specific antisense lncRNA in *trans* in a dose-dependent manner, while interference with the lncRNA of an active *var* gene down-regulated its expression and induced rapid switching to other *var* genes (1). Interestingly, a specific RNA degrading enzyme called PfRNase II has been proposed to target the antisense lncRNAs and has been linked to regulation of type A *var* genes, thus providing additional support for a role for these transcripts in *var* gene activation (103). Parasites deficient for PfRNase II displayed overexpression of both full-length mRNAs and intron-derived antisense lncRNAs from type A *var* genes, which have been linked to severe disease (46, 77). In addition, the activated gene was expressed simultaneously with a type C *var* gene, indicating that monoallelic expression was disrupted (103).

In addition to the sense and antisense lncRNAs that are transcribed from *var* genes, additional lncRNAs are transcribed from genomic elements close to some *var* genes. Early genome sequencing projects identified unusual GC-rich elements within *var* gene tandem arrays (16, 40, 93). These elements display near complete sequence identity, appear to contain RNA pol III promoter

elements and transcribe RNAs that were detected in later transcriptomic analyses (12, 64, 68, 84). Recently, it was shown that expression of these transcripts is clonally variant and was associated with derepression of the flanking *var* locus (99). This observation was supported by FISH data indicating that the GC-rich lncRNAs colocalize with both subtelomeric and internal *var* genes (42) and are linked to *var* gene activation. However, experiments using reporter genes indicated an opposite, transcriptionally repressive effect linked to heterochromatin propagation (99). The mechanism by which these transcripts might influence chromatin structure needs further investigation, though it seems unlikely that a transcript so highly conserved in sequence could directly be involved in the "choice" for activation of an individual *var* gene, which requires gene specificity.

A ROLE FOR RNA POLYMERASE II IN EPIGENETIC REGULATION

Studies in model eukaryotes have revealed that in addition to its familiar role in generating mRNAs, RNA Pol II is also a major player in epigenetic regulation and imprinting. During transcription elongation, the C-terminal domain (CTD) recruits numerous factors involved in regulating the transcription cycle, premRNA splicing, and mRNA export, but also histone modifiers that influence the epigenetic imprint at the transcribed locus (17, 66). This concept is particularly intriguing for *var* gene regulation since RNA pol II produces three distinct RNAs from *var* genes: 1) an mRNA transcribed from the upstream promoter of a single gene at a time, 2) antisense lncRNAs transcribed from an intron promoter from the single active gene, and 3) sense lncRNAs transcribed from the introns of all *var* genes (**Figure 2*a*,*b***). Further, these activities differ depending on whether a gene is active or silent. The first experimental evidence for a possible role for active transcription by RNA pol II in maintaining the transcriptional state of a *var* gene came from a series of experiments in which RNA pol II recruitment to the upstream promoter of the active *var* gene was temporarily prevented through competition with a high copy number, episomal *var* upstream region (29). This "promoter titration" experiment led to a complete loss of epigenetic memory. These data are consistent with a requirement for RNA Pol II recruitment and transcription through the locus for maintenance of the epigenetic marks found at the active *var* gene. RNA pol II is also actively recruited to the intron of each *var* gene where it transcribes sense lncRNAs late in the asexual cycle, from all *var* genes regardless if they are active or silent (50, 87). As described previously, experiments with reporter gene constructs indicate that this promoter activity is required for proper *var* gene regulation (23, 38), and interestingly, an alternative RNA pol II promoter can substitute for a *var* intron in these assays (31), suggesting that it might be the recruitment of RNA pol II itself that is responsible for the influence of *var* introns on *var* gene expression. Lastly, RNA pol II also transcribes the previously described antisense lncRNAs (50), initiated within *var* introns and extending into the 3′ end of exon I. This activity is only detected at the single active *var* gene and happens early in the replicative cycle (1, 47), unlike the sense lncRNAs that are made late in schizogony.

The association of different factors with RNA Pol II depends on post-transcriptional modifications of the CTD, primarily acetylation and phosphorylation of serine residues within a tandem array of heptad repeats of the sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser/Lys7 (32). Similar to model organisms, the CTD of RNA Pol II in *Plasmodium* spp. undergoes phosphorylation on Ser2 and Ser5, indicating that it also likely recruits histone modifiers and plays a role in influencing the epigenetic state of the genome (74). In higher eukaryotes the phosphorylation pattern of the CTD changes depending whether the polymerase is transcribing mRNA or ncRNAs (33), and the phosphorylation pattern determines which histone modifiers are recruited (13, 34, 57). RNA pol II therefore likely recruits different sets of histone modifiers to *var* loci depending on whether it is producing mRNA, antisense lncRNA or sense lncRNA, and the interplay of the resulting

chromatin marks could contribute to epigenetic memory and *var* gene switching (**Figure 2*c*,*d***). Consistent with this model, the CTD of RNA Pol II of *P. falciparum* can directly bind to and recruit the histone modifier PfSET2, which deposits the mark H3K36me3, one of the histone marks known to be important for *var* gene regulation (91). PfSET2 binding to the CTD was shown to be directly dependent on its phosphorylated state (91), and disruption of this interaction profoundly alters *var* gene expression patterns (92), reinforcing the role of RNA Pol II in recruiting epigenetic factors to *var* loci. The fact that H3K36me3 marks both active and silent *var* genes implicates recruitment of PfSET2 by RNA pol II during transcription of sense lncRNAs from *var* introns rather than during mRNA transcription at the single, active *var* gene. Similarly, this mark might be involved in "recognition" of the *var* gene family, rather than transcriptional silencing or activation per se.

One cannot exclude the possibility that in addition to recruitment of chromatin modifying enzymes by RNA Pol II, the lncRNA molecules themselves could play a role in marking a locus through incorporation into the chromatin fiber and recruitment of enzymes for epigenetic imprinting. Interestingly, the conserved intronic region of the *var* antisense transcript contains the complementary sequence of the insulator-like "pairing elements" found to be essential for the interactions between *var* upstream promoters and introns that contribute to silencing (1, 3). These elements were shown to bind specific nuclear protein complexes that have not been yet characterized. It is tempting to propose a model by which the incorporation of the antisense lncRNAs into chromatin could prevent binding of protein complexes to the pairing elements, either through competition or by direct nucleic acid hybridization, thereby disrupting the interaction between intron and the *var* promoter and activating the gene. Alternatively, the conserved domain of these antisense lncRNAs could directly recruit proteins involved in epigenetic imprinting to maintain epigenetic memory. Further experimental data will gain better insight on the mechanisms by which these antisense lncRNAs contribute to activation of a single *var* gene.

HOW DOES MUTUALLY EXCLUSIVE EXPRESSION WORK?

Although considerable insights have been obtained in recent years regarding activation and silencing of individual members of the *var* gene family, one question that continues to perplex the field is the concept of mutually exclusive expression. How does a cell manage an entire gene family such that one gene and only one gene is transcriptionally active at a time? Further, how is a switching event coordinated such that as the previously active gene is silenced a new gene is simultaneously activated? Several models have been proposed, but none has yet to gain acceptance within the field. It is also worth noting that mutually exclusive *var* gene expression is consistently observed by many laboratories; however, occasional examples of parasites expressing more than one *var* gene or no *var* genes at all have been described (4, 26, 48), suggesting that some flexibility in the system might exist.

Consistent with the previously mentioned idea that *var* introns could contribute to *var* gene recognition by recruitment of PfSET2 through transcription of sense lncRNAs by RNA pol II, disruption of the gene encoding PfSET2 completely disrupts mutually exclusive expression, leading to expression of the entire gene family simultaneously (47). In addition, the pseudogene *var1csa* (the single type D *var* gene) has a substantial deletion within its intron that disrupts RNA pol II recruitment and lncRNA production. This gene has been reported to not be subject to mutually exclusive expression (51, 101). As described above, experimental knockdowns of the histone modifier PfHda2 and PfHP1 both result in disruption of mutually exclusive expression and lead to simultaneous expression of the entire *var* gene family (10, 19). However, it is not clear from these experiments if these two proteins are required for mutually exclusive expression per se,

or if rather these knockdown lines are simply unable to silence any genes, thus causing all *var* genes to become transcriptionally active. It is worth noting that the gene *Pfapi2-g*, which is not a member of the *var* gene family but contains the same "pairing elements" and is similarly silenced through the assembly of heterochromatin, is also desilenced in these transgenic lines, suggesting that these knockouts lead to a general loss of heterochromatin based silencing rather than a specific disruption of mutually exclusive *var* gene expression.

The ability to activate multiple *var* promoters simultaneously through various genetic manipulations negates several models for mutually exclusive expression that have been proposed in other systems. For example, a long-distance enhancer (or locus control region) might interact with and activate a single *var* promoter at a time as was previously suggested for regulating allelic exclusion of the genes encoding olfactory receptors in mammals (54). However, the ability to activate multiple *var* promoters simultaneously, either in rare cases where two *var* genes have been observed to be simultaneously transcribed (26, 48) or in episomal studies in which many *var* promoters are transcriptionally active (29, 31, 95), would appear to exclude this model. These studies similarly discount a mechanism based on limited access to an exclusive subnuclear expression site as was proposed to regulate exclusive expression of *vsg* genes in African trypanosomes (65). In the intestinal parasite *Giardia lamblia*, an RNAi or miRNA-based mechanism is thought to maintain allelic exclusion of the *vsp* family through either the selective degradation of mRNAs from all members of the family but one or by translational repression (73, 81, 82). However, *Plasmodium* lacks the enzymes required for RNAi or miRNA-based control of targeted mRNAs and thus cannot utilize this mechanism to control *var* gene expression (9). Lastly, unlike other systems of mutually exclusive expression that employ a feedback mechanism to ensure proper protein expression (olfactory gene expression, immunoglobulin production, etc.), production of PfEMP1 is not essential for proper regulation of a *var* gene expression (30, 95). Instead, gene regulation appears to act at the level of transcription alone and depends on interactions between the noncoding regulatory regions that surround each *var* gene. Thus while much has been learned about *var* gene activation and silencing, the mechanistic basis for mutually exclusive expression remains an enigma.

IS SWITCHING SOMEHOW COORDINATED?

Historic experiments utilizing experimental human infections have shown that *P. falciparum* can maintain a chronic infection that can persist for up to a year or more (61). These infections are typified by waves of antigenically distinct populations of parasites with each wave presumably representing parasites expressing a single (or very few) *var* gene. More recent field studies have similarly found that parasites isolated from specific tissues of patients express a very small number of *var* genes (63, 89). The parasitemia in any given wave can be upwards of 10^5 parasites per μl of blood, indicating a total parasite load of trillions of parasites. Given the enormous number of individual parasites in the circulation of an infected individual at any given time, how the parasite population as a whole can display a seemingly coordinated and timely display of different PfEMP1s over the course of an infection is completely unknown. There is no evidence for a specific order of *var* gene expression, and there remain no data indicating communication between individual parasites that influence *var* gene switching. Thus, the mechanism underlying these patterns of switching is not understood. It has been shown that the different *var* gene types display different activation rates in cultured parasites, with the type A and B genes showing faster switching rates than the more stable type C genes (37, 67). However, these observations alone do not provide a solution to this problem.

Recker and Newbold addressed this question by applying mathematical modeling to datasets derived from chronic, experimental human infections (76). Their analysis led them to propose a

model in which expression switching is coordinated through the utilization of a specific *var* gene that serves as a "sink node" within a switching network. Thus all switching events are initiated by the transient activation of the gene that occupies this node. After activating the node, expression can return to the originally active *var* gene (a nonswitching event) or alternatively expression can deviate to a previously silent *var* gene (switching expression to an alternative PfEMP1). Thus, all parasites within the population routinely return to the same starting point within the *var* gene network. According to their analysis, this mathematical model accurately explains the waves of parasitemia observed in experimental infections.

While the "sink node" model lacks molecular evidence, it does make several predictions that can be investigated. If one or more sink nodes exist (and they are in fact *var* genes), they would be predicted to be conserved in all parasite isolates, unlike the rest of the gene family that is hypervariable when comparing different parasite isolates. Three such *var* genes exist: type D (*var1csa*), type E (*var2csa*) and the so-called type 3 *var* genes (51, 97, 101, 104). Additionally, a *var* gene acting as a transiently expressed node should not lead to recognition by the immune system, especially if it is activated repeatedly over the course of an infection. In this respect *var2csa* is particularly intriguing. It has been demonstrated to have a second layer of regulation that can repress translation of the mRNA, thus preventing expression of PfEMP1 and thereby avoiding the immune response (2, 6, 62). Lastly, experiments using either genetic manipulation or low doses of histone methyltransferase inhibitors that induce *var* gene switching result in the selective activation of *var2csa* (91, 92). Whether the sink node model (and a role for *var2csa* in coordinating switching) proves to be partially or fully accurate awaits further experimentation. However, it remains the only fully described model that attempts to explain this puzzling phenomenon.

ADVANCES IN TECHNOLOGY AND FUTURE STUDIES

The study of the molecular basis for antigenic variation in malaria parasites can borrow from breakthroughs in the study of other infectious organisms that undergo similar processes, including bacterial and fungal pathogens (25). Although some aspects are clearly shared, e.g., a role of epigenetics and chromatin structure, other details differ significantly. For example, chromatin modifiers demonstrated to play a key role in regulating *var* gene expression in *P. falciparum* are completely absent from malaria parasites that infect nonprimate hosts (91), and there is evidence that epigenetic memory might not play a role in antigenic variation in rodent malaria parasites (22). As greater amounts of genome data from evolutionarily distant parasites accumulate, the power of comparative genomics can be applied to this topic. Further advances in technology are also likely to provide openings for additional breakthroughs. One technical difficulty for studying *var* gene regulation is the limitation of being forced to measure transcription at the population level, which makes it difficult to differentiate between experiments that disrupt mutually exclusive expression and those that lead to very rapid switching. Rapidly advancing technologies that allow single cell analysis should help circumvent this problem and enable researchers to pinpoint the mechanisms involved. In addition, the ability to look at nuclear dynamics in vivo in greater resolution, using state of the art super resolution microscopy, for example STORM and PALM or STED, will greatly improve our ability to understand the nuclear processes involved in *var* gene regulation. Lastly, the adaptation of CRISPR based methods to malaria parasites is already having a tremendous effect on the field (41, 96). Genetic manipulation of parasites is now more rapid and precise, greatly improving how quickly experiments can be completed. Cumulatively, these advances will undoubtedly continue to shed light on this fascinating biological problem.

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