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Malaria Epigenetics

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Organisms with identical genome sequences can show substantial differences in their phenotypes owing to epigenetic changes that result in different use of their genes. Epigenetic regulation of gene expression plays a key role in the control of several fundamental processes in the biology of malaria parasites, including antigenic variation and sexual differentiation. Some of the histone modifications and chromatin-modifying enzymes that control the epigenetic states of malaria genes have been characterized, and their functions are beginning to be unraveled. The fundamental principles of epigenetic regulation of gene expression appear to be conserved between malaria parasites and model eukaryotes, but important peculiarities exist. Here, we review the current knowledge of malaria epigenetics and discuss how it can be exploited for the development of new molecular markers and new types of drugs that may contribute to malaria eradication efforts.

Epigenetic regulation of gene expression refers to heritable changes in transcription that occur in the absence of alterations in the primary sequence of DNA. Numerous important biological pathways in eukaryotes involve epigenetic regulation, both in health and during disease. Chromatin is the main platform where epigenetic processes take place, such that epigenetic traits are typically mediated by DNA modifications (such as methylation) or by changes in chromatin structure such as histone posttranslational modifications or use of histone variants. In fact, there is an accepted "relaxed" use of the term epigenetics that includes all chromatin-based processes that affect transcription, regardless of whether or not information is transmitted through cell division.

In malaria parasites, epigenetic regulation of gene expression has been extensively studied only in *Plasmodium falciparum*. For many years, studying epigenetics in this parasite was almost synonymous to studying the regulation of *var* genes, which are important for antigenic variation and virulence (Kyes et al. 2001). However, recent findings have revealed a more general role for epigenetics in malaria parasite biology, including processes as diverse as erythrocyte invasion, solute transport, or formation of sexual forms necessary for human-to-mosquito transmission. The contribution of epigenetic regula-

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tion of gene expression to these processes stems from the clonally variant expression of some of the genes involved. Silencing of clonally variant genes, which is a process truly controlled at the epigenetic level (Cortés et al. 2012), generally depends on histone modifications that result in reversible formation of repressive chromatin structures (heterochromatin), but several additional layers of regulation operate specifically on particular gene families such as *var* genes.

Genome-wide studies of heterochromatin marks or transcriptional variation identified many genes regulated at the epigenetic level that are not involved in the processes mentioned above, indicating that epigenetic regulation of gene expression plays a role in the control of other as-yet-uncharacterized biological processes (Flueck et al. 2009; Lopez-Rubio et al. 2009; Rovira-Graells et al. 2012). In the years to come, the biological significance of epigenetic regulation of these genes should be revealed. In all cases, epigenetic changes are likely to imply translating the same genome into alternative transcriptomes and phenotypes, increasing the plasticity of parasite populations and favoring their survival.

PROCESSES REGULATED AT THE EPIGENETIC LEVEL IN MALARIA PARASITES

Numerous *P. falciparum* genes involved in host–parasite interactions show clonally variant expression, such that they are expressed in some individual parasites, but not in others that are genetically identical (Table 1). The active or silenced states of these genes are clonally transmitted during asexual replication, with switches between the two states occurring at low frequencies. The predicted functions for clonally variant genes suggest that transcriptional variation results in both antigenic and functional differences at several stages along the parasite's life cycle (Fig. 1), but the phenotypic alterations associated with changes in the expression of specific clonally variant genes have been elucidated in only a few cases. In this section, we describe the known processes in which epigenetic changes determine alternative phenotypes. A common theme is that, for processes in which the parasite requires alternative operational states to adapt to changes in its environment or to evade host immune responses, alternative epigenetic states exist for some of the genes linked to the process.

Antigenic Variation and Cytoadherence

Observations of both human and animal infections clearly show that malaria parasites are capable of maintaining persistent, chronic infections for extended periods of time, even in the presence of a robust antibody response of the infected host. In experimental human infections, parasites have been observed to persist for over a year, frequently displaying characteristic waves of parasites reminiscent of similar population dynamics displayed by other infectious organisms including African trypanosomes (Miller et al. 1994). Thus, it is clear that malaria parasites are capable of showing systematic antigenic variation that results in immune evasion, presumably through alterations made to proteins displayed on the surface of infected erythrocytes. In the case of *P. falciparum*, the formation of knobs on the infected erythrocyte membrane as well as the induction of cytoadherent properties of infected cells provided additional evidence for the placement of antigenically variant molecules on the infected cell surface (Miller et al. 2002). The variant surface properties of the infected erythrocytes were linked to epigenetic regulation of gene expression with the discovery and description in *P. falciparum* of the *var* multicopy gene family and the multitude of alternative forms of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) that it encodes (Baruch et al. 1995; Smith et al. 1995; Su et al. 1995).

Individual parasites typically express only one *var* gene at a time, a pattern known as mutually exclusive expression that operates on top of a clonal variation and results in a single form of PfEMP1 on the erythrocyte surface. All variants of PfEMP1 include a single transmembrane domain that passes through the erythrocyte membrane, thus anchoring a long, hypervariable portion of the protein that binds to endothelial surface receptors and thereby

Table 1. Examples of genes or gene families that display clonally variant expression linked to the histone modification H3K9me3 (when transcriptionally silent) in *Plasmodium falciparum*

Gene ID (previous ID)	Protein name	Proposed function	Notes	References
PF3D7_1222600 (PFL1085w)	PfAP2-G	Transcription factor implicated in sexual differentiation	Protein contains an AP2 DNA-binding domain	Kafsack et al. 2014
PF3D7_1036300 (PF10_0355)	MSPDBL2	A likely merozoite surface protein, possibly involved in erythrocyte invasion	Gene under strong balancing selection Possible role in drug resistance	Amambua-Ngwa et al. 2012; Hodder et al. 2012; Van Tyne et al. 2013
PF3D7_1301600 (MAL13P1.60)	EBA140	Merozoite protein involved in erythrocyte invasion, binds to glycophorin C	Enables parasites to use alternative invasion pathways	Maier et al. 2003; Cortés et al. 2007; Crowley et al. 2011
PF3D7_0424200 (PFD1150c)	PfRH4	Merozoite protein involved in erythrocyte invasion, binds to complement receptor 1 (CR1)	Enables parasites to use alternative invasion pathways	Stubbs et al. 2005; Jiang et al. 2010
var gene family (approximately 60 members)	PfEMP1	Cytoadhesive protein displayed on the surface of the infected erythrocyte	Displays mutually exclusive expression, changes in expression enable antigenic variation	Chookajorn et al. 2007; Lopez-Rubio et al. 2007; Jiang et al. 2013
rif gene family (approximately 160 members)	RIFIN	Displayed on the surface of the infected erythrocyte, some members of the family have been linked to rosetting	Most members of the family silent at any given time, expression has also been reported in merozoites	Kyes et al. 1999; Fernandez et al. 1999; Mwakalinga et al. 2012; Goel et al. 2015
stevor gene family (approximately 31 members)	STEVOR	Displayed on the surface of the infected erythrocyte; contribute to infected erythrocyte mechanical properties	Most members of the family silent at any given time, expression has also been reported in merozoites and gametocytes	Lavazec et al. 2007; Sanyal et al. 2012; Niang et al. 2014
Pfmc-2tm gene family (approximately 12 members)	PFMC-2TM	Displayed on the surface of the infected erythrocyte	Most members of the family silent at any given time	Sam-Yellowe et al. 2004; Lavazec et al. 2007
surfin gene family (approximately 10 members)	SURFIN	Unknown function	Several localizations reported including infected erythrocyte surface and merozoites	Winter et al. 2005; Mphande et al. 2008
clag gene family (five members)	CLAG	At least some members are implicated in the formation of the PSAC channel found in the infected erythrocyte membrane	*clag3.1* and *clag3.2* are expressed in a mutually exclusive fashion, whereas *clag2* shows independent clonally variant expression	Cortés et al. 2007; Comeaux et al. 2011; Crowley et al. 2011; Nguitragool et al. 2011

Continued

Table 1. *Continued*

Gene ID (previous ID)	Protein name	Proposed function	Notes	References
phist gene families (approximately 79 members)	PHIST-domain proteins	Cytoadherence/erythrocyte remodeling	Various families of exported proteins; at least some PHIST-domain proteins interact with PfEMP1 at knobs	Sargeant et al. 2006; Proellocks et al. 2014; Oberli et al. 2014
exported dnaj III gene family (approximately nine members)	DnaJ III/HSP40	Erythrocyte remodeling	Exported proteins with a J-domain; HSP40-type chaperones	Sargeant et al. 2006
hyp gene families (approximately 55 members)	HYP	Unknown function	Various families of exported proteins	Sargeant et al. 2006
fikk gene family (approximately 23 members)	FIKK	Erythrocyte remodeling	Exported kinases	Sargeant et al. 2006; Nunes et al. 2007; Kats et al. 2014
gbp gene family (three members)	Glycophorin binding protein (GBP)	Unknown	Exported proteins	(Sargeant et al. 2006)
acs gene family (approximately 13 members)	Acyl-CoA synthetase (ACS)	Lipid metabolism (synthesis and/or transport)	Gene family specifically amplified in *P. falciparum*	Bethke et al. 2006
acbp gene family (four members)	Acyl-coA binding protein (ACBP)	Lipid metabolism (synthesis and/or transport)	Not characterized	Bethke et al. 2006

Inclusion of a gene or gene family in this table is based on the direct observation of clonally variant expression (Rovira-Graells et al. 2012) and the presence of epigenetic marks of silencing (Flueck et al. 2009; Lopez-Rubio et al. 2009) in genome-wide studies, or in the specific references provided. Both current 3D7 ID numbers and previous ID numbers are provided, along with a brief, general description of the predicted function. The estimates of the number of genes in each family do not include pseudogenes.

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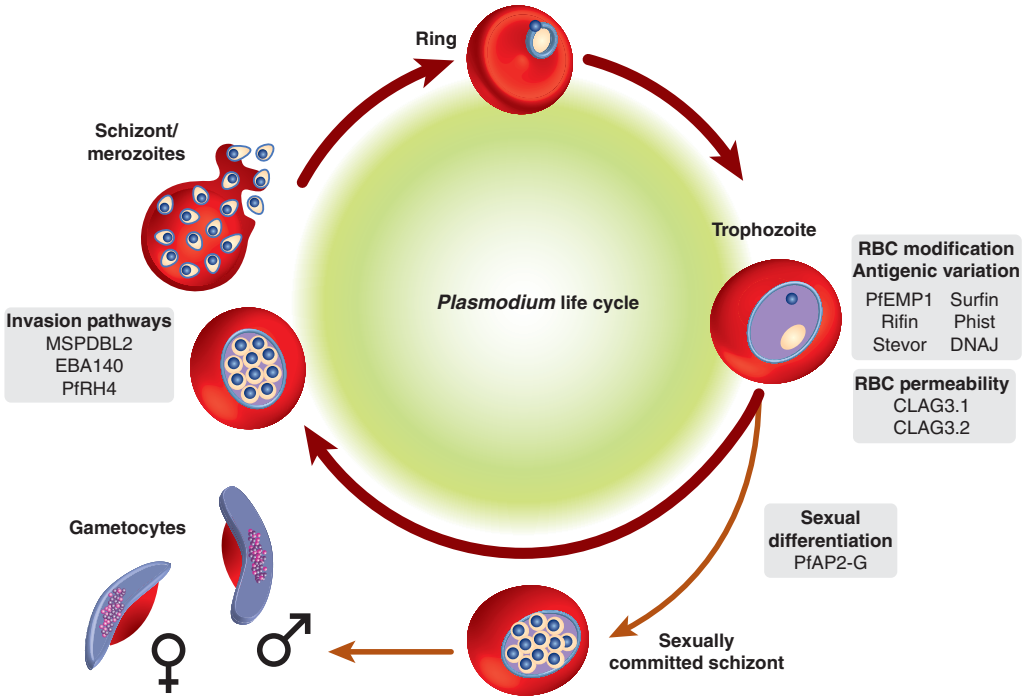

Figure 1. Clonally variant gene expression during the blood stages of the *Plasmodium falciparum* life cycle. Asexual replication occurs within human erythrocytes and progresses through different morphological stages, including rings, trophozoites, schizonts, and merozoites (cycle at *top* of figure). In trophozoites, several clonally variant proteins involved in erythrocyte modification, antigenic variation, and cell permeability are expressed. Similarly, in schizonts/merozoites, clonally variant proteins that determine alternative erythrocyte invasion pathways are expressed. In a small proportion of cells, the sexual differentiation process is initiated through the activation of PfAP2-G, leading to the production of male and female gametocytes (*bottom*).

mediates cytoadhesion. Cytoadhesion of infected erythrocytes effectively removes them from the peripheral circulation, a phenomenon referred to as sequestration, thereby enabling them to avoid passage through the spleen where they would be detected and destroyed. The different forms of PfEMP1 display different binding specificities, utilizing alternative host endothelial surface ligands for adhesion. This results in parasitized erythrocytes sequestering within different host tissues depending on which *var* gene (and the encoded form of PfEMP1) is expressed (Montgomery et al. 2007). Sequestration within the brain and the placenta has been directly linked to cerebral malaria and pregnancy-associated malaria, respectively, thus making PfEMP1 the best-characterized virulence factor of malaria caused by *P. falciparum*. PfEMP1 also mediates rosetting, the binding of infected to noninfected erythrocytes that is also associated with virulence (Miller et al. 2002).

With the availability of complete genome sequences for several *Plasmodium* species infecting both primates and rodents, it has become possible to catalogue the many multicopy gene families found within these genomes. Gene families encoding variant surface proteins have been discovered in multiple *Plasmodium* species, suggesting that this is a universal characteristic of malaria parasites. Although some of these gene families are conserved throughout the *Plasmodium* lineage, others appear to be species-specific. In addition to the *var* gene family, which includes approximately 60 members per haploid genome, *P. falciparum* also possesses several other large, multicopy gene families like-

ly involved in antigenic variation. These include the *rif*/*stevor*/*Pfmc-2tm* (approximately 200 copies) and the surfins (approximately 10 copies) (Weber 1988; Kyes et al. 1999; Sam-Yellowe et al. 2004; Winter et al. 2005). The largest multicopy gene family in *P. vivax* is referred to as *vir* (approximately 350 copies) (del Portillo et al. 2001), which appears to have orthologous gene families in the primate parasite *P. knowlesi* (*kir*) and in the rodent parasites *P. berghei* (*bir*), *P. yoelii* (*yir*), and *P. chabaudi* (*cir*) (Fischer et al. 2003; Janssen et al. 2004) and is also likely a distant ortholog of the *rif* genes of *P. falciparum* (Janssen et al. 2004). *Plasmodium knowlesi* also possesses the *sica-var* family (approximately 100 copies), which encodes a protein linked to cytoadherence similar to PfEMP1 (al-Khedery et al. 1999). In all cases, expression is limited to one or a limited subset of the members of each gene family at any given time. Activation and silencing of the genes of these families, as well as switching expression between members, are all thought to be regulated epigenetically.

Erythrocyte Invasion

Invasion of erythrocytes is an essential step of the asexual cycle of malaria parasites. After schizonts burst, merozoites are released into the bloodstream and quickly invade new erythrocytes to start a new cycle of replication. Erythrocyte invasion involves several differentiated steps, starting with initial contact, followed by merozoite reorientation and apical attachment that lead to the formation of a moving junction that progresses until the merozoite is internalized (Wright and Rayner 2014; Weiss et al. 2015). The small *eba* and *Pfrh* gene families, each including four to five members, encode adhesins that are released from apical organelles and interact with erythrocyte receptors to mediate merozoite reorientation/apical attachment. Of note, merozoites can use alternative pathways for this step of invasion, each involving a different set of erythrocyte receptors. With the exception of *Pfrh5*, the genes of the *eba* and *Pfrh* families are nonessential and appear to be functionally redundant (Reed et al. 2000; Duraisingh et al. 2003; Maier et al. 2003; Stubbs et al. 2005; Wright and Rayner 2014). As expected from the existence of alternative invasion pathways and the nonessentiality of these genes, several *eba* and *Pfrh* genes are spontaneously silenced in some parasite lines, and clonally variant expression mediated by epigenetic mechanisms has been directly shown in some cases (Taylor et al. 2002; Duraisingh et al. 2003; Cortés et al. 2007; Jiang et al. 2010; Crowley et al. 2011). Importantly, variant expression of invasion adhesins has also been observed in field isolates (Cortés 2008; Gomez-Escobar et al. 2010). Although the relation between *eba* and *Pfrh* expression patterns and the use of specific invasion pathways is complex and not completely understood, it is generally accepted that changes in the expression of these genes lead to phenotypic variation in the process of invasion (Duraisingh et al. 2003; Cortés 2008; Wright and Rayner 2014).

Understanding what selective advantage the clonally variant expression of invasion adhesins confers to parasites is an unresolved question. Although flexibility in front of genetic variability in host erythrocytes is an appealing possibility, mutations in the majority of erythrocyte receptors that *P. falciparum* uses for invasion are rare, especially in Africa. Immune evasion provides an alternative explanation for the potential advantage that variant expression of invasion ligands may confer to the parasites. The number of genes in the *eba* or *Pfrh* families is small for "classic" antigenic variation, but variant expression may act synergistically with genetic polymorphism among different isolates to evade acquired immune responses (Cortés 2008; Wright and Rayner 2014).

The proteins encoded by some members of the variantly expressed large multigene families *rif*, *stevor*, and *surfin* discussed above also appear to be expressed in merozoites (Chan et al. 2014), although their function in the merozoite remains unknown and may be unrelated to invasion. Another gene encoding a protein expressed in merozoites that shows clonally variant expression is *mspdbl2*, a member of the small *msp3-like* family. MSPDBL2 is located at the merozoite surface, where it may play a role in the initial steps of erythrocyte invasion. This

intriguing gene, which carries the epigenetic marks of silencing associated with clonally variant gene expression (Flueck et al. 2009; Lopez-Rubio et al. 2009), appears to be silenced in the vast majority of parasites and activated in only small subpopulations (~1% of parasites in most lines) (Amambua-Ngwa et al. 2012). However, the phenotypic differences resulting from the alternative transcriptional states of *mspdbl2* remain unknown.

Infected Erythrocyte Permeability

The transport of many solutes across the infected erythrocyte membranes is mediated by the plasmodial surface anion channel (PSAC), a broad selectivity channel with several unusual transport properties (Desai 2014). Recent research has established that the product of the parasite genes *clag3.1* or *clag3.2* is required for the formation of functional PSAC and efficient nutrient acquisition (Nguitragool et al. 2011; Pillai et al. 2012). This was an unexpected finding because these genes were previously thought to participate in either cytoadherence or erythrocyte invasion (Gupta et al. 2015).

The link between solute uptake and epigenetics comes from studies demonstrating that *clag3* genes show clonally variant expression regulated at the epigenetic level (Cortés et al. 2007; Comeaux et al. 2011; Crowley et al. 2011). Interestingly, *clag3* genes generally show mutually exclusive expression, such that individual parasites express either *clag3.1* or *clag3.2* (Cortés et al. 2007). However, it appears to not be strict, with occasional parasites escaping mutual exclusion (Rovira-Graells et al. 2015). Although the driving force for the epigenetic regulation of the expression of *clag3* genes remains unclear, it is reasonable to hypothesize that expression of alternative *clag3* genes may result in different solute transport phenotypes, which may play a role in the adaptation of the parasite to varying concentrations of nutrients and other solutes in the host plasma. This might explain why mutually exclusive expression need not be strict because silencing or expressing both genes simultaneously could provide additional phenotypic plasticity to parasite populations. In support of this view, challenging *P. falciparum* cultures with the toxic compound blasticidin S, which requires the PSAC for transport across the infected erythrocyte membrane, results in the selection of parasites with dramatic switches in *clag3* expression (Mira-Martínez et al. 2013; Sharma et al. 2013). This is indicative of a role for *clag3* epigenetic regulation in the trade-off between excluding toxic compounds from infected erythrocytes and allowing the entrance of nutrients necessary for normal growth. These results also show that switches in *clag3* expression constitute a novel antimalarial drug resistance mechanism controlled at the epigenetic level. Finally, CLAG3 proteins are exposed on the erythrocyte surface (Nguitragool et al. 2011), so in addition to influencing solute transport, switches in the expression of *clag3* genes may also play a role in antigenic variation and immune evasion.

Sexual Conversion

The complex life cycle of malaria parasites includes multiple well-differentiated stages through which the parasite progresses in an ordered manner. The single developmental decision for the parasite occurs during asexual growth in the bloodstream: at each cycle of replication, the parasite makes a choice between continuing asexual multiplication, as the majority of parasites do, and irreversibly converting into male or female gametocytes, which are the sexual forms of the parasite necessary for transmission to another host via a mosquito vector (Fig. 1).

Genome-wide studies on transcriptional variation and epigenomic studies identified a member of the ApiAP2 family of transcriptional regulators that shows clonally variant expression (Rovira-Graells et al. 2012) and carries epigenetic marks of silencing (Flueck et al. 2009; Lopez-Rubio et al. 2009). The characterization of this transcriptional regulator, termed PfAP2-G, revealed that it plays an essential role in triggering sexual differentiation, such that the gene is by default silenced by heterochromatin-based epigenetic mechanisms, and only the few parasites in which the gene is stochastically activated

convert into gametocytes (Kafsack et al. 2014). These results raised the idea that sexual conversion is regulated at the epigenetic level, a view that was later corroborated by studies in which specific epigenetic factors were depleted (Brancucci et al. 2014; Coleman et al. 2014). The ortholog of *pfap2-g* in the distantly related murine malaria parasite *P. berghei*, *pbap2-g*, also plays a key role in gametocyte formation (Sinha et al. 2014). This observation suggests that *ap2-g* is a conserved regulator of sexual conversion in malaria parasites; whether or not epigenetic control of the process is a conserved feature in all *Plasmodium* species awaits experimental confirmation.

The proportion of parasites that convert into sexual forms varies among isogenic parasite subclones (Kafsack et al. 2014). Spontaneous sexual conversion rates in these subclones are stable, indicating that as-yet-uncharacterized epigenetic mechanisms transmit the probability of *pfap2-g* activation and subsequent sexual conversion. Additionally, sexual conversion rates are affected by some environmental conditions (Bousema and Drakeley 2011), which opens up exciting perspectives about the possibility that environmental cues influence *pfap2-g* epigenetic states. This would provide one of the first examples in malaria of the parasite sensing the state of its host and producing a directed transcriptional response.

Epigenetic Variation and Adaptation to Environmental Changes: Bet-Hedging

Clonally variant expression is an intrinsic property of many *P. falciparum* gene families involved in disparate cellular processes (Rovira-Graells et al. 2012). This implies that isogenic parasite populations spontaneously become transcriptionally heterogeneous during normal growth, such that different individual parasites have different combinations of active and epigenetically silenced genes. This diversity confers plasticity to parasite populations because it provides the grounds for dynamic natural selection of parasites with highest fitness as changes in the environment occur. Adaptive strategies based on the stochastic generation of phenotypic diversity within populations before any challenge occurs, as opposed to directed adaptive responses, are commonly referred to as bet-hedging (Veening et al. 2008; Simons 2011).

The high level of spontaneous transcriptional diversity observed within isogenic parasite populations and the apparently stochastic nature of expression switches in clonally variant genes support the view that malaria parasites commonly use bet-hedging adaptive strategies (Rovira-Graells et al. 2012). This idea is also supported by the proposed limited ability of *P. falciparum* to adapt via directed transcriptional responses (Ganesan et al. 2008; Le Roch et al. 2008), although this remains controversial (Deitsch et al. 2007). In addition to the processes described above, in which epigenetic changes can be ascribed to alterations in specific phenotypes, the predicted functions of some of the clonally variant genes identified (Table 1) make it tempting to speculate that they modulate fitness in front of the majority of conditions that commonly fluctuate in the human blood, which is the environment where *P. falciparum* asexual stages reside. However, altogether there are few examples of well-defined adaptive pathways to specific challenges via bet-hedging in malaria, so it is still unclear whether epigenetic variation and bet-hedging play a general role in the adaptation of malaria parasite populations to changes in their environment. More experimental insight into the association between epigenetic diversity and long-term fitness in natural environments, and the identification of the clonally variant genes that mediate adaptation to specific changes in the parasite environment, are clearly needed to determine the actual contribution of bet-hedging to malarial adaptation.

EPIGENETIC MECHANISMS IN *Plasmodium falciparum*

Given the important role that epigenetic regulation of gene expression plays in the biology of malaria parasites, considerable work has focused on determining the molecular mechanisms that underlie these processes. The lion's share of this work has been performed with *P. falciparum*, although additional work has

also been performed in the rodent models of malaria. In this section, we summarize recent studies that shed light on the molecular basis for epigenetic regulation in *P. falciparum*.

Chromatin Structure across the Life Cycle

As mentioned briefly above, epigenetic regulation of gene expression involves heritable modifications to the way the genome is packaged, which influence gene activation and silencing. Many of these modifications occur on histones, the protein subunits that comprise the nucleosomes around which the DNA strands of the chromosomes are wrapped. Modifications that increase the affinity of the histones to the DNA, thereby resulting in a more condensed chromatin structure that is less accessible to transcription complexes (called heterochromatin), lead to silencing of gene expression, whereas those that result in more open chromatin (called euchromatin) are associated with active regions of the genome. Typical nucleosome modifications include the incorporation of histone variants or posttranslational modifications (most often acetylation or methylation) to the amino-terminal "tails" of histones H3 and H4. Many of the enzymes that catalyze the addition or removal of these modifications have been identified encoded in the *P. falciparum* genome and some have been experimentally characterized (Table 2). Once formed, heterochromatin and euchromatin are separated within the nucleus, with heterochromatin generally found segregated to the nuclear periphery. In *P. falciparum*, as in other eukaryotes, many regions of the genome are found in either a euchromatic or a constitutively heterochromatic state that is the same in all cells, indicating that the chromatin state in such regions is likely dictated by the underlying primary DNA sequence. Although constitutive heterochromatin is, in general, transcriptionally silent, some long noncoding RNAs (lncRNAs) are expressed from subtelomeric repeats heterochromatin (Sierra-Miranda et al. 2012; Broadbent et al. 2015). However, other regions of the genome can be found in alternative chromatin states in different individual cells (Fig. 2). In such regions, known as facultative heterochromatin or bistable chromatin, once one or the other state is established, the resulting gene expression patterns are heritable through multiple cell generations, a phenomenon frequently referred to as epigenetic memory. However, these states are reversible, thus a gene that is silenced for many generations can once again revert to the active state, and vice versa. Herein lies the mechanistic basis for clonally variant expression and the biological phenomena described above.

Progression through the different stages of the asexual cycle of *P. falciparum* requires a tightly regulated cascade of gene expression in which most of the 5300 genes display a narrowly defined timing of activation (Bozdech et al. 2003; Le Roch et al. 2003). The general chromatin structure of the genome as a whole similarly displays cyclical, dynamic changes. The density of nucleosomes along the length of the chromosomes, called nucleosome occupancy, is somewhat depleted as cells enter S phase in the trophozoite stage, then becomes maximal in schizonts (Bunnik et al. 2014). Nucleosomes also appear to be clearly positioned around transcriptional landmark sites, including transcription start sites, regulatory elements, and splice donor and acceptor sites (Kensche et al. 2016). Euchromatic upstream regulatory regions of most genes are typically associated with the presence of the histone variants H2A.Z and H2B.Z, acetylation of histone H3 on the lysine in the ninth position (H3K9ac) and methylation of the lysine in the fourth position (H3K4me3). The presence of both of these histone modifications shows dynamic changes over the course of the asexual cycle, such that H3K9ac levels directly correlate with temporal patterns of gene expression, whereas H3K4me3 increases throughout the genome from low levels in rings to highly enriched levels late in the cycle (Lopez-Rubio et al. 2009; Salcedo-Amaya et al. 2009; Bartfai et al. 2010; Hoeijmakers et al. 2013). Other histone modifications similarly correlate with transcript levels, and their incorporation into the chromatin at specific regions of the genome appears to depend on the underlying DNA sequence (Gupta et al. 2013). All of these dynamic chromatin changes that occur over the course of the asexual cycle

Table 2. List of putative epigenetic factors involved in controlling chromatin structure and epigenetic regulation in *P. falciparum*

Protein name	Gene ID (previous ID)	Proposed function	References
Histone methyltransferases			
PfSET1	PF3D7_0629700 (PFF1440w)	Involved in the deposition of the epigenetic mark H3K4me3	Cui et al. 2008a
PfSET2 (also called PfSETvs)	PF3D7_1322100 (MAL13P1.122)	Involved in the deposition of the epigenetic mark H3K36me2/3, participates in *var* regulation	Cui et al. 2008a; Kishore et al. 2013; Jiang et al. 2013; Ukaegbu et al. 2014
PfSET3 (also called PfKMT1)	PF3D7_0827800 (PF08_0012)	Involved in the deposition of the epigenetic mark H3K9me2/3	Cui et al. 2008a; Lopez-Rubio et al. 2009; Volz et al. 2010
PfSET4	PF3D7_0910000 (PFI0485c)	Involved in the deposition of epigenetic marks on H3K4	Cui et al. 2008a; Volz et al. 2010; Jiang et al. 2013
PfSET5	PF3D7_1214200 (PFL0690c)	Involved in the deposition of unknown epigenetic marks; mitochondrial localization also reported	Cui et al. 2008a; Volz et al. 2010; Jiang et al. 2013
PfSET6	PF3D7_1355300 (PF13_0293)	Involved in the deposition of epigenetic marks on H3K4	Cui et al. 2008a; Volz et al. 2010
PfSET7	PF3D7_1115200 (PF11_0160)	In vitro data suggest methylation of H3K4 and H3K9	Cui et al. 2008a; Chen et al. 2016
PfSET8	PF3D7_0403900 (PFD0190w)	Involved in the deposition of the epigenetic mark H4K20me1/2/3	Cui et al. 2008a; Kishore et al. 2013; Jiang et al. 2013
PfSET9	PF3D7_0508100 (PFE0400w)	Involved in the deposition of unknown epigenetic marks	Cui et al. 2008a
PfSET10	PF3D7_1221000 (PFL1010c)	Involved in the deposition of the epigenetic mark H3K4me3, localized to the *var* expression site	Volz et al. 2012
Histone demethylases			
JmjC1	PF3D7_0809900 (MAL8P1.111)	Involved in the removal of epigenetic marks from H3K9 and H3K36	Cui et al. 2008a; Jiang et al. 2013
JmjC2	PF3D7_0602800 (PFF0135w)	Involved in the removal of unknown epigenetic marks	Cui et al. 2008a; Jiang et al. 2013
LSD1	PF3D7_1211600 (PFL0575w)	Involved in the removal of unknown epigenetic marks	Iyer et al. 2008; Volz et al. 2010; Jiang et al. 2013
Histone acetyltransferases			
PfGCN5	PF3D7_0823300 (PF08_0034)	Involved in the deposition of the epigenetic marks H3K9ac and H3K14ac	Fan et al. 2004; Cui et al. 2007
PfHAT1	PF3D7_0416400 (PFD0795w)	Probable ortholog to HAT1 in higher eukaryotes	
PfMYST	PF3D7_1118600 (PF11_0192)	Member of the MYST family of acetyltransferases, proposed to acetylate H4K5, K8, K12, and K16	Miao et al. 2010
Histone deacetylases			
PfSIR2A PfSIR2B	PF3D7_1328800 (PF13_0152) PF3D7_1451400 (PF14_0489)	Involved in telomere maintenance and regulation of *var* gene expression	Duraisingh et al. 2005; Freitas-Junior et al. 2005; Tonkin et al. 2009; Merrick et al. 2015

Continued

Table 2. *Continued*

Protein name	Gene ID (previous ID)	Proposed function	References
PfHDAC1	PF3D7_0925700 (PFI1260c)	Putative class I histone deacetylase, probable ortholog of Rpd3 from yeast	Joshi et al. 1999; Andrews et al. 2012b
PfHDAC2	PF3D7_1472200 (PF14_0690)	Putative class II histone deacetylase	Andrews et al. 2012b
PfHDAC3 (also called PfHda2)	PF3D7_1008000 (PF10_0078)	Putative class II histone deacetylase, linked to *var* gene silencing and sexual differentiation	Andrews et al. 2012b; Coleman et al. 2014
Other			
PfBDP1	PF3D7_1033700 (PF10_0328)	Bromodomain protein 1, involved in the regulation of genes linked to erythrocyte invasion	Josling et al. 2015
PfHP1	PF3D7_1220900 (PFL1005c)	Heterochromatin protein 1, involved in the maintenance of silenced regions of the genome, linked to *var* gene silencing and sexual differentiation	Perez-Toledo et al. 2009; Flueck et al. 2009; Brancucci et al. 2014

Both current 3D7 ID numbers and previous numbers are provided, along with a brief, general description of the predicted function. Many of the listed functions are predicted based on computational analysis and have not been experimentally verified. Several additional uncharacterized putative epigenetic factors have been predicted by in silico analysis (Bischoff and Vaquero 2010).

within constitutively euchromatic regions of the genome are unlikely to carry heritable information (Cortés et al. 2012), but together they contribute to the cascade of gene activation that enables the parasites to faithfully complete each round of schizogony. Special chromatin also forms at centromeres, including the incorporation of the variant histone cenH3. The centromeres from the different chromosomes cluster together within the nucleus during mitosis and cytokinesis, but dissociate after the merozoites reinvade erythrocytes and reinitiate a new cycle (Hoeijmakers et al. 2012). Additionally, DNA methylation has also been detected and proposed to contribute to transcriptional regulation (Ponts et al. 2013).

General Mechanisms Regulating Clonally Variant Gene Expression

As mentioned above, genes that display clonally variant expression are associated with bistable chromatin, which can be found as either euchromatin or facultative heterochromatin, resulting in either activation or silencing, respectively. This chromatin state is heritable and, thus, must be faithfully reproduced as the genome transitions through multiple rounds of replication and division during schizogony. Only relatively rarely does a gene switch transcriptional states; however, the ability to switch is key for parasites to be able to display clonally variant expression and is indispensable for processes like antigenic variation that depend on generating variability over time, and for bet-hedging strategies that enable parasite populations to respond to changes in their environment. In addition to switching transcriptional states, some genes also belong to gene families in which only one or a small number of genes are active at a time (frequently referred to as mutually exclusive expression). Thus, activation of one gene necessitates the simultaneous silencing of the previously active member of the family, therefore requiring a mechanism of coordination within the family.

Significant progress has been made in recent years in identifying and characterizing the epigenetic components associated with activation and silencing of individual genes. Much less is

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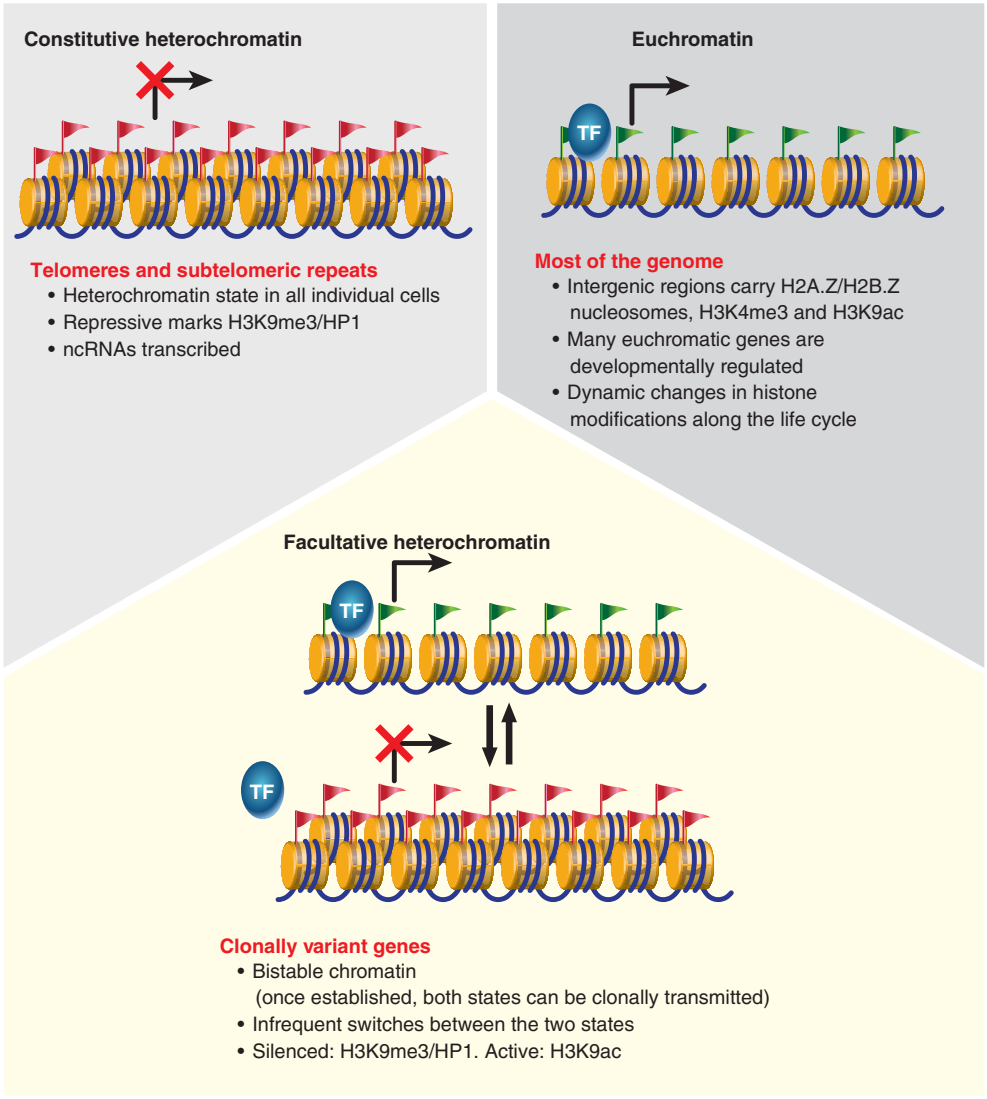

Figure 2. Chromatin compartments in *Plasmodium falciparum*. As in other eukaryotes, the chromatin of malaria parasites can be roughly divided into three separate compartments with very distinct properties. Green and red flags represent histone marks generally associated with transcriptional activation or silencing, respectively. *P. falciparum* constitutive heterochromatin (*upper left*) is generally transcriptionally silent but allows transcription of some noncoding RNAs (ncRNAs). Euchromatic regions (*upper right*) are typified by the incorporation of the variant histones H2A.Z and H2B.Z as well as the histone modifications H3K4me3 and H3K9ac. Stage-specific transcription largely depends on the presence of specific transcription factors (TFs), whereas in facultative heterochromatin (*bottom*) transcription depends on both the presence of the relevant transcription factor(s) and chromatin accessibility. The latter is determined by which of the possible chromatin states has been assembled at a specific locus in a given cell. Typically, chromatin at silent loci incorporates the histone modification H3K9me3 and is bound by HP1, whereas active genes are associated with the histone modification H3K9ac.

understood regarding what controls switching between transcriptional states, and the molecular basis for mutually exclusive expression within gene families remains entirely mysterious. The clonally variant genes involved in antigenic variation (*var*, *rifin*, *stevor*, *Pfmc-2tm*), alternative erythrocyte invasion pathways (*eba* and *Pfrh*), infected erythrocyte permeability (*clag3.1* and *clag3.2*), and sexual conversion (*pfap2-g*) all share certain epigenetic characteristics. When they are transcriptionally silent, they are bound by nucleosomes that incorporate the silent mark H3K9me3, in particular surrounding the transcriptional start site (Chookajorn et al. 2007; Lopez-Rubio et al. 2009; Jiang et al. 2010; Crowley et al. 2011; Kafsack et al. 2014). When individual genes are activated, H3K9me3 is replaced by H3K9ac, and this appears to be a key step in switching the transcriptional state of the gene (Lopez-Rubio et al. 2007; Crowley et al. 2011). Once in place, the activating or silencing histone marks are maintained throughout the remainder of the asexual cycle, even at points in the cycle when the genes are inactive. Furthermore, these marks are faithfully reproduced as the genome replicates through schizogony, resulting in epigenetic memory. Changes in these histone modifications occur at low frequencies, resulting in clonal variation. The H3K9me3 modification is recognized by heterochromatin protein 1 (HP1) that begins the chromatin condensation process that results in segregation into the nuclear periphery and prevents transcription (Flueck et al. 2009; Perez-Toledo et al. 2009). The importance of HP1 in this process is shown by experiments in which its expression is disrupted. In the absence of HP1, all clonally variant genes become transcriptionally active simultaneously, leading to disruption of antigenic variation and rapid differentiation into the sexual conversion pathway (Brancucci et al. 2014). A similar phenotype results from disruption of the histone deacetylase PfHda2 (Coleman et al. 2014), further demonstrating the shared epigenetic mechanisms controlling expression of clonally variant gene families. These profound phenotypes reveal the importance of proper regulation of heterochromatin for parasite biology.

Specific Mechanisms Regulating *var* Gene Expression

Of the genes that undergo clonally variant expression, the most work has focused on the *var* gene family that encodes the variant surface protein PfEMP1. The haploid genome of any given parasite includes approximately 60 *var* genes, which are expressed in a mutually exclusive manner. As described above, the active gene is associated with chromatin that incorporates the H3K9ac modification, whereas the remaining, silent members of the family are marked by histones carrying H3K9me3. As expected, the silent genes are bound by HP1 and incorporated into heterochromatin that is segregated into the nuclear periphery, in which they group together into five to seven clusters (Freitas-Junior et al. 2000). Interestingly, the active member of the family is also found at the nuclear periphery, but it is separated from the silent genes into a subnuclear position that also includes the histone methyltransferase PfSET10 (Duraisingh et al. 2005; Ralph et al. 2005; Volz et al. 2012). It is postulated that this methyltransferase is responsible for the H3K4me3 modification found at the active *var* gene (Volz et al. 2012). An additional histone mark that appears to be important for *var* gene regulation is H3K36me3, a modification incorporated by the methyltransferase PfSET2 (Cui et al. 2008a) that in model eukaryotes is typically associated with transcriptional elongation. Surprisingly, disruption of the gene encoding PfSET2 completely disrupts mutually exclusive expression, leading to simultaneous expression of all members of the family and suggesting that H3K36me3 might be required for *var* gene silencing (Jiang et al. 2013). However, H3K36me3 is found within the body of both active and silent *var* genes, suggesting an alternative model in which this histone mark is required for the recognition of *var* genes as members of the *var* gene family. This hypothesis has implications for coordination of gene expression and mutually exclusive expression.

In addition to specific histone modifications associated with active and silent *var* genes, incorporation of variant histones into the nucle-

osomes found at specific regions of the genome also appears to contribute to *var* gene regulation. The variant histone H2A.Z is found within nucleosomes throughout the genome wherever H3K9ac and H3K4me3 are found (Bartfai et al. 2010; Petter et al. 2011), consistent with a role in maintaining euchromatin. Although at most genes incorporation of H2A.Z is stable throughout the asexual cycle, at the single active *var* gene H2A.Z incorporation into nucleosomes at the promoter appears to be temporally regulated and is observed only at the point in the cycle when the *var* gene is actively transcribed (Petter et al. 2011), thus nucleosome exchange could play a role in *var* gene activation. In contrast, silent *var* genes are devoid of this histone variant. More recent work has identified double-variant histones that incorporate both H2A.Z and H2B.Z at transcriptionally active genomic regions, including the active *var* gene promoter (Hoeijmakers et al. 2013; Petter et al. 2013). The amount of double-variant histones found within intergenic regions correlates with the strength of the nearby promoters as well as the base composition of the underlying DNA, suggesting a model in which double-variant histones help to demarcate transcriptionally active and silent regions of the genome.

Although the histone marks associated with active or silent *var* genes have now been identified, it is not yet clear how the histone modifiers are selectively targeted to specific genes to properly mark them for activation or silencing. DNA regulatory elements have been identified both within individual *var* genes (Avraham et al. 2012; Brancucci et al. 2012) and separating genes within *var* tandem arrays (Wei et al. 2015), but their possible role in recruiting epigenetic factors remains uncharacterized. ncRNAs have been implicated in this process in many model eukaryotic systems, and similar mechanisms are likely to be at play with *var* genes. ncRNAs were first identified associated with *var* genes when this gene family was initially discovered, although their function was not understood (Su et al. 1995). These RNAs are transcribed from an RNA pol II promoter found within the conserved intron located within all *var* genes (Kyes et al. 2007). The intron is required for the recognition of *var* genes for mutually exclusive expression (Deitsch et al. 2001; Dzikowski et al. 2007), and it has been shown to transcribe an antisense ncRNA from the single active gene while producing sense ncRNAs from all members of the family (Jiang et al. 2013; Amit-Avraham et al. 2015). The role of the ncRNAs themselves is not known; however, targeting the antisense transcripts for degradation or overexpression greatly alters *var* gene expression patterns (Amit-Avraham et al. 2015), and an RNA exosome was recently identified that appears to be important for controlling antisense ncRNA levels (Zhang et al. 2014). Disruption of the RNAse activity of this exosome strongly affects *var* gene expression patterns. In addition to the ncRNAs themselves, the RNA pol II complex that transcribes them might also play a direct role in recruiting histone modifiers to *var* loci. PfSET2, the histone methyltransferase that deposits the H3K36me3 mark at *var* genes, binds to the carboxy-terminal domain of RNA pol II and, thus, could be recruited by the polymerase while it is transcribing the sense ncRNAs (Ukaegbu et al. 2014). This would explain why both active and silent genes are marked by H3K36me3, and is consistent with the hypothesis that this mark is responsible for genes to be recognized as members of the *var* gene family. Additional work will begin to decipher how these different aspects of *var* gene regulation integrate into an overall mechanism of control, and further how mutually exclusive expression and switching are coordinated. Work in the field will also help to determine how *var* gene switching integrates with selection by the human immune system (Abdi et al. 2016), a topic that has not been extensively studied, and also how transmission through the mosquito potentially "resets" the expression patterns of clonally variant gene families (Spence et al. 2015).

IMPORTANCE OF RESEARCH ON MALARIA EPIGENETICS IN THE CONTEXT OF ERADICATION

The new paradigm in the fight against malaria is eradication. An agenda has been developed to guide the steps that need to be undertaken to

achieve this ambitious aim (Alonso et al. 2011). Considering the low expectations of having a highly effective vaccine available in the next few years, elimination efforts will have to rely mainly on vector control strategies and antimalarial drugs that could be used in mass drug administration schemes. Monitoring and diagnostic tools will also play important roles in elimination campaigns. Research on malaria epigenetics can contribute to the development of new public health tools that could facilitate elimination.

Monitoring Malaria Phenotypes: Genes under Epigenetic Regulation as Markers for Virulence and Transmission Potential

Malaria elimination campaigns will represent an extremely strong selective pressure for malaria parasites, which makes it reasonable to fear that such interventions may result in accelerated parasite evolution. There is a risk that elimination efforts may select for "super-parasites" with undesirable traits, such as increased virulence, more efficient transmission, increased resilience in front of low vector availability, increased resistance to multiple drugs, or even an increased ability to generate resistance to new drugs reminiscent of the "accelerated resistance to multiple drugs" (ARMD) phenotype (Rathod et al. 1997). Monitoring how parasite traits evolve during elimination campaigns is essential to avoid potentially disastrous consequences of the attempts to eradicate the disease. Monitoring will be necessary at the epidemiological level, but also at the genetic, phenotypic, and epigenetic levels. Strong pressures can obviously result in the selection of genetic variants that confer increased fitness in front of the new challenges, but it is also possible that there is a selection of parasites with epigenetic patterns that have an analogous effect. As described above, some parasite traits are associated with the expression of specific clonally variant genes: for example, expression of some *var* genes is associated with increased risk of severe disease (Jensen et al. 2004; Rottmann et al. 2006; Avril et al. 2012; Claessens et al. 2012; Lavstsen et al. 2012), and *pfap2-g* expression levels could be used as a proxy for parasite investment into sexual conversion and transmission. Expression of these genes and several others should be characterized in parasites that persist in settings in which elimination efforts are not completely successful and a residual parasite population survives, albeit with altered epidemiology. This could help to understand the adaptive pathways of surviving parasites, as well as to appreciate the potential risks associated with the evolved parasites (e.g., increased virulence), or to identify their Achilles' heels.

Epigenetic Regulation of Gene Expression as a Target for Therapeutic Intervention: "Epidrugs"

It is unclear whether existing drugs, used wisely, are sufficient to achieve success in malaria elimination campaigns. In any case, new antimalarial drugs with desirable properties, such as being effective against all parasite stages or requiring a single dose, if available, would certainly facilitate the task. In this regard, epigenetic regulators are considered a promising new class of drug targets, with some attractive characteristics described below.

Epigenetic regulation of gene expression is a highly dynamic process, implying that it is possible to alter normal epigenetic states through modulation of the enzymes involved in the process. Interfering pharmacologically with the delicate balance between the alternative epigenetic states of clonally variant genes could compromise parasite survival in several ways (Fig. 3). First, inhibiting the epigenetic factors that are necessary for *var* silencing could result in parasites that simultaneously express multiple forms of PfEMP1, inducing the development of broadly reactive immune responses that eliminate the current infection and additionally confer protection against future infections. An analogous strategy to disrupt normal antigenic variation has been successfully applied to induce protective immunity against *Giardia lamblia* in rodent models of disease (Rivero et al. 2010). Second, altering the balance between the active and silenced states of *pfap2-g* could result in either massive sexual conversion or no pro-

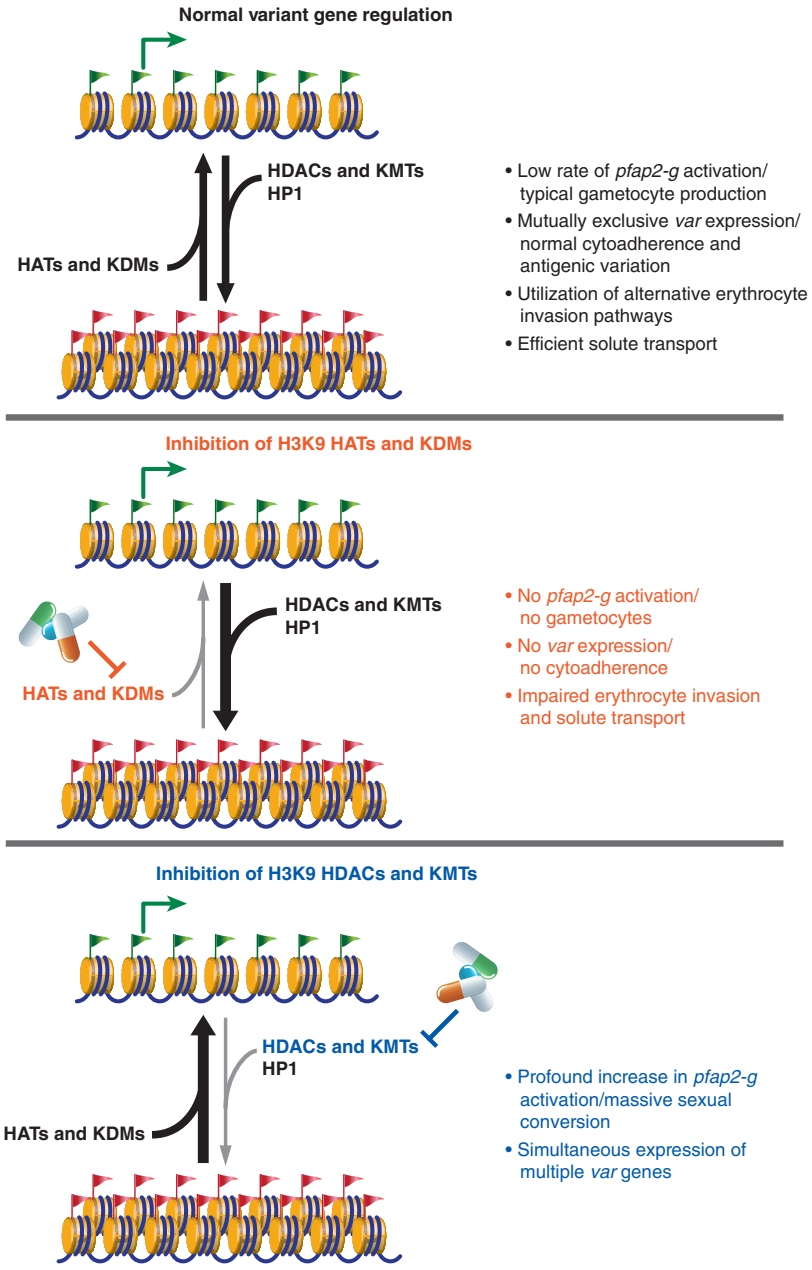

Figure 3. Epigenetic factors as targets for drug development. The normal balance between the euchromatic (active) and heterochromatic (silenced) states of clonally variant genes (*top* panel) can be altered pharmacologically. Drugs that inhibit the factors that catalyze the transition to or the maintenance of the active state are expected to shift the balance toward the silenced state (*middle* panel). In contrast, drugs that inhibit the factors that catalyze the transition to or the maintenance of the silenced state are expected to shift the balance toward the active state (*bottom* panel). The predicted effects of chemical inhibition of enzymes that participate in the regulation of clonally variant genes in general are listed. As examples, enzymes that operate on H3K9 are shown, but inhibition of enzymes that regulate the deposition or removal of other histone modifications (e.g., H4K20me3) may have similar effects. Inhibitors of epigenetic factors that participate in the regulation of only some clonally variant gene families (e.g., *var* genes) are expected to have family-specific effects. The predicted histone acetyltransferases (HATs), lysine demethylases (KDMs), histone deacetylases (HDACs), and lysine methyltransferases (KMTs) that operate on H3K9 are described in Table 2.

duction of sexual forms, both with catastrophic consequences for the parasite. It is important to note that malaria elimination efforts would tremendously benefit from drugs that kill gametocytes and consequently prevent transmission, but gametocytes are highly resilient to chemical attack and most current antimalarial drugs are not efficient against them (malERA Consultative Group on Drugs 2011). Thus, inhibiting the epigenetic mechanisms that drive the conversion of parasites into sexual forms arguably provides an attractive alternative to directly targeting gametocytes. Third, inhibiting malaria chromatin modifiers that do not specifically regulate clonally variant genes but participate in normal cell cycle progression could directly kill the parasites.

Orthologs of many known chromatin modifiers have been identified in the *P. falciparum* genome (Cui et al. 2008a; Bischoff and Vaquero 2010). The first obvious targets for antimalarial "epidrug" development are the enzymes that add or remove acetyl or methyl groups from histone tails (Table 2). Importantly, inhibitors of this type of enzymes have been developed for the fight against other diseases such as cancer, providing a large number of chemical starting points and a wealth of knowledge that could be used for the development of malaria-specific epigenetic inhibitors (Arrowsmith et al. 2012). Several compounds that were identified as inhibitors of histone deacetylases (HDACs) or acetyltransferases (HATs) in other eukaryotes inhibit *P. falciparum* growth, and some of them have a more potent effect on malaria parasites than on human cells (Merrick and Duraisingh 2007; Cui et al. 2008b; Andrews et al. 2012a,b; Duffy et al. 2014). Inhibitors of *P. falciparum* lysine methyltransferases (KMTs) have also been identified and shown to effectively kill malaria parasites of different species and at different stages of development (Malmquist et al. 2012, 2015). Furthermore, a sublethal concentration of one such compound, chaetocin, was recently shown to alter the epigenetic regulation of *var* gene expression by increasing the frequency of expression switches in this gene family (Ukaegbu et al. 2015). Interestingly, another KMT inhibitor was shown to activate dormant liver forms called hypnozoites (Dembele et al. 2014), which are produced by some malaria parasite species including *P. vivax* and are considered one of the less accessible malaria infection reservoirs. This observation raises the intriguing possibility that hypnozoite activation may be regulated at the epigenetic level. If this is the case, it may be possible to target these highly resilient forms with epigenetic drugs.

FUTURE PERSPECTIVES

Epigenetics continues to be a vibrant field of investigation in all eukaryotic systems, from animals to plants to protozoans. Given their somewhat "stripped down" repertoire of transcription factors, it is likely to play an even bigger role in the biology of malaria parasites. With the development of drugs that target epigenetic factors as therapies for human diseases like autoimmune disorders or cancer, this rapidly evolving technology can be applied to the development of antimalarial compounds that kill parasites, reduce virulence, and disrupt transmission, all key components of any elimination/eradication campaign. Along the way, these compounds will also serve as tools for investigating the basic biology of these evolutionarily distant and fascinating organisms. The recent development of new technologies for genetic manipulation of the parasites, including powerful methods for genome editing, will also contribute to addressing some of the burning questions in the malaria epigenetics field described in this chapter. Considering that epigenetics often studies properties that vary from one cell to another, it will be important to also develop improved technologies for analysis at the single-cell level, which could bring enormous progress to the field.

CONCLUDING REMARKS

It is exciting to once again consider the prospects of malaria elimination and eradication after such plans were previously abandoned decades ago. Recent gains in reducing the global malaria burden provide tantalizing hope that elimination is indeed possible. Nonetheless,

for progress against the disease to be sustained, new intervention strategies will be required as drug and insecticide resistance inevitably arise. As funds and resources are devoted to disease reduction in the field, it will be important not to neglect basic research into the fundamental biology of the parasite and its mosquito vector. It is this discovery process that will yield the drugs, vaccines, and intervention strategies of the future that will be required for the ultimate eradication of malaria. Epigenetics is one such field that is rapidly developing and likely to be a rich source of novel targets for antimalaria strategies.

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