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Invited Review

Genomics and epigenetics of sexual commitment in *Plasmodium*

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ABSTRACT

Malaria is the disease caused by the apicomplexan parasites belonging to the genus *Plasmodium*. Expanding our arsenal to include transmission-blocking agents in our fight against malaria is becoming increasingly important. Such an implementation requires detailed understanding of the biology of the *Plasmodium* life cycle stages that are transmissible. *Plasmodium* gametocytes are the only parasite stage that can be transmitted to the mosquito vector and are the product of sexual development in a small percentage of parasites that continually proliferate in host blood. The critical decision made by asexual erythrocytic stages to cease further proliferation and differentiate into gametocytes, as well as the first steps they take into maturity, have long remained unknown. Recent studies have contributed to a breakthrough in our understanding of this branch point in development. In this review, we will discuss the findings that have allowed us to make this major leap forward in our knowledge of sexual commitment in *Plasmodium*. We will further propose a model for the mechanism triggering the switch to sexual development, constructed around the proteins currently known to regulate this process. Further insight into sexual commitment and gametocyte development will help identify targets for the development of transmission-blocking malaria therapies.

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1. Introduction

Malaria is a mosquito-borne parasitic disease caused by apicomplexan protozoa of the genus *Plasmodium*. Human malaria is caused by five *Plasmodium* spp. and has a dramatic health and economic impact worldwide. The most lethal of these, *Plasmodium falciparum*, is responsible for approximately 500,000 deaths annually, largely in sub-Saharan Africa, disproportionately affecting the poor, children under 5 years old and pregnant women. Despite the fact that control interventions over the last decades have saved the lives of millions, the disease remains an intractable problem, with approximately 214 million new cases reported in 2015 (World Health Organization, 2015).

The success of malaria parasites could be attributed to their complex life cycle, comprising a range of asexual and sexual developmental stages, as well as their sheer numbers coupled with wide genetic diversity among and within populations (Manske et al., 2012). To date, anti-malarial agents have largely targeted the pathology-causing, asexual, blood stages of the parasites, often ineffectively clearing sexual stages and allowing transmission from asymptomatic hosts (Bousema et al., 2006; Butterworth et al., 2013; Miller et al., 2013; Abdul-Ghani et al., 2015). Furthermore, the obligatory phase of sexual reproduction in the mosquito midgut is not only prerequisite for transmission, but also enables meiotic recombination between genomes, with the consequent production of parasites with re-assorted genotypes. It results in new combinations of polymorphic genes, thus facilitating evolutionary adaptation and the emergence and spread of drug resistance (reviewed in Babiker and Walliker, 1997).

For these reasons, attention of the scientific community is shifting to include development of transmission-blocking agents (reviewed in Alonso et al., 2011). Transmission is initiated by sexual commitment that leads to the production of mature circulating gametocytes and it comprises a series of processes: gametocyte activation, gamete fertilisation, ookinete development and progression through the midgut wall, generation of oocysts (the sites of sporogony) and sporozoite migration to the salivary gland. Infective sporozoites are then delivered into the host during the blood meal taken by the mosquito, eventually colonising the liver and initiating the mammalian phase of the parasite life cycle. Following hepatocyte rupture, parasites enter the circulation, where the potential for replication is vast. Processes relating to transmission represent biological bottlenecks of varying severity, resulting in a substantial reduction in the number of parasites (reviewed in Sinden, 2010; Wu et al., 2015). Thus, targeting these bottleneck

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processes would reduce transmission from symptomatic and asymptomatic hosts, it would prevent sexual recombination within the vector, decreasing the likelihood of drug resistance spreading, and would also provide an efficient strategy in the fight to eliminate the disease as smaller populations can be targeted much more efficiently.

Blocking transmission to the anopheline vector requires disruption of the production or functionality of gametocytes, the gamete precursors and the only sexual stage developing in host blood. Since the initial description of gametocytes in 1880, their morphology and metabolic profiles have been examined and there has been progress in identifying genes involved in their differentiation (reviewed in Alano, 2007; Dixon et al., 2008; Baker, 2010). However, many aspects of their biology remain unknown, including the initiation of their development. Intriguingly, only a small proportion of the parasites per erythrocytic cycle commit to gametocytogenesis (Ponnudurai et al., 1982; Graves et al., 1984). All merozoites from a sexually committed schizont develop into either male or female gametocytes, a developmental decision made at a point prior to the release of the committed merozoites (Silvestrini et al., 2000). Once committed, the *P. falciparum* gametocyte develops over 10–12 days through five distinct morphological stages (I-V), the last of which is taken up by the feeding mosquito (Josling and Llinás, 2015). Gametocytes of other *Plasmodium* spp. develop much more quickly, albeit typically slightly slower than the asexual stage (Galinski et al., 2013).

The mechanism underlying commitment to sexual differentiation has until recently remained elusive, but there is now evidence that it is an epigenetically regulated process. Epigenetic regulation allows differential gene expression during development, the pattern of which can be inherited via DNA or histone post-translational modifications (PTMs), without alteration of the nucleotide sequence (Jaenisch and Bird, 2003). A *Plasmodium* genome has a typical nucleosomal organization (Fig. 1A) and epigenetic phenomena are indeed mediated by PTMs of histones, replacement of core histones by histone variants (H2A, H2B, H3, H4 by H2A.Z, H2B.Z, H3.3, CenH3, respectively) and chromatin remodelling (Fig. 1B) (Cui and Miao, 2010; Duffy et al., 2012; Voss et al., 2014). While the biological importance of DNA methylation as an epigenetic modification has been widely recognised, only recently has there been evidence provided that it is likely involved in regulating gene expression in *Plasmodium*, through the identification of a functional DNA cytosine methyltransferase and genome-wide mapping of methylated cytosines in *P. falciparum* (Ponts et al., 2013).

Epigenetic regulation is critical in many processes in *Plasmodium* biology, including the demarcation of functional elements in the genome and life cycle progression (reviewed in Merrick and Duraisingh, 2010; Cortés et al., 2012). Control at the epigenetic level also regulates clonally variant gene expression, a process that results in transcriptional heterogeneity among a clonal parasite population. This spontaneous transcriptional switching of certain genes and gene families, also known as bet-hedging, offers a method of adaptation that is alternative to traditional gene regulation. It is key for the success of the parasite as it generates a phenotypically heterogeneous population, thus making the parasite fitter in response to varied environmental challenges, before these even present. Several genes are variantly transcribed and contribute to bet-hedging, including the main high-copy gene families and, most importantly, the *var* gene family (Rovira-Graells et al., 2012). This family encodes for *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), the antigens responsible for sequestration of infected red blood cells (RBCs) in the microvasculature and persistence of infection. An intricate epigenetic strategy, which involves reversible histone modifications, chromatin remodelling and gene repositioning, regulates monoallelic expression of the 60-member *var* gene family and results in antigenic variation of PfEMP1 (Guizetti and Scherf, 2013).

Renewed interest in transmission blocking interventions underlies the importance of deciphering sexual commitment in *Plasmodium*. Recent studies have contributed to a breakthrough in our understanding of this branch point in development; a transcription factor (TF) essential for commitment to gametocytogenesis has been elucidated, which, in turn, provided the basis for unravelling the role epigenetics plays in this process. In this review we will discuss the recent findings that have allowed the development of a model for the mechanism triggering the switch to sexual development. The model is derived from research on *P. falciparum*, the blood stages of which are readily cultured in vitro, and *Plasmodium berghei*, a rodent parasite which provides an in vivo model.

2. AP2-G, the master regulator of gametocytogenesis

The identification of AP2-G as a regulator of sexual differentiation in *Plasmodium* by two independent studies has provided a key piece of the unresolved puzzle of commitment to gametocytogenesis (Kafsack et al., 2014; Sinha et al., 2014). AP2-G is part of the apiAP2 family of DNA binding proteins, which belongs to the larger Apetala2/ethylene response factor protein family found in apicomplexan protists and *Plantae* (Balaji et al., 2005; Campbell et al., 2010). Many of the ApiAP2 members characterised to date regulate major developmental transitions throughout the *Plasmodium* life cycle (Balaji et al., 2005; Iyer et al., 2008; Campbell et al., 2010). *Pfap2-g* stood out as the most variantly transcribed member of the AP2 family in a study analysing transcriptional heterogeneity within several clonal parasite populations. Its detection among differentially transcribed genes linked to early gametocyte development (Rovira-Graells et al., 2012) and its conservation throughout the phylum was consistent with a role in commitment to sexual development.

Forward genetic approaches which identified *ap2-g* as the only mutated gene in a number of gametocyte non-producing (GNP) *P. falciparum* and *P. berghei* lines strengthened this hypothesis (Kafsack et al., 2014; Sinha et al., 2014). Evidently, *ap2-g* inactivation has repeatedly occurred in vitro and after continuous blood passage. However, no loss-of-function mutations in the specific locus have been found in approximately 300 *P. falciparum* field isolates, where generation of gametocytes is required for transmission (Kafsack et al., 2014). The link between AP2-G and the ability to produce gametocytes was corroborated by the generation of deletion mutants (Δpfap2-g and Δpbap2-g), both of which lost their ability to create gametocytes. Genetic complementation of Δpbap2-g and correction of the mutations in the previously identified *P. berghei* GNP lines rescued the phenotype (Sinha et al., 2014). Attempts to generate complementation constructs were unsuccessful for Δpfap2-g, but Kafsack et al. (2014) verified the link between *pfap2-g* deletion and the Δpfap2-g phenotype by creation of a PfAP2-G knockdown line using the destabilisation domain (DD) system (Banaszynski et al., 2006). Accumulation of PfAP2-G, as a result of addition of the shield compound, lead to a 30-fold increase in gametocytogenesis relative to the control compound-treated parasites, restoring the levels of commitment to those observed in the high-gametocyte-producing parental line (Kafsack et al., 2014).

The potential role of AP2-G as a transcriptional regulator of sexual-specific gene expression was substantiated by comparison of the transcriptome of Δpfap2-g and its parent line. Downregulated genes included many expressed in the initial stages of gametocytogenesis and some of the known early markers of sexual commitment (*pfs16*, *pfg27/25* and *pfg14.744*) (Kafsack et al., 2014). Comparative microarray analysis of *pbap2-g* deficient

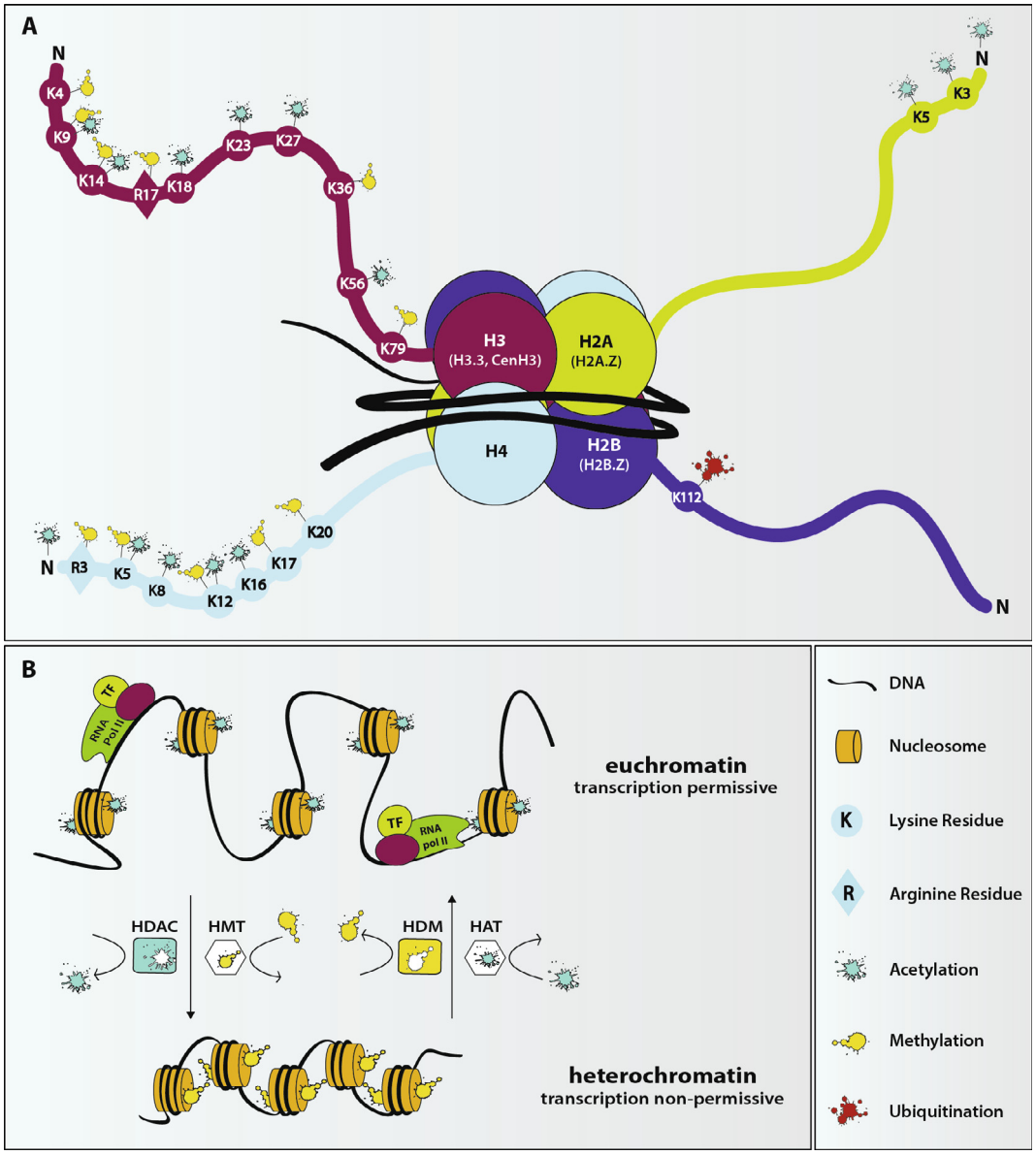

Fig. 1. *Plasmodium falciparum* gene regulation by histone post-translational modifications and chromatin landscape alterations. (A) The canonical histones, depicted in their octamer nucleosomal organization, contain covalent post-translational modifications on their N terminal tail, the majority of which are histone lysine acetylation and methylation marks. Histone acetylation is associated with gene activity and histone methylation with both activity and silencing dependent on the residue methylated. These tail post-translational modifications are maintained by various histone acetyltransferases, deacetylases (HDACs), methyltransferases, demethylases (HDMs) and proteins with post-translational modification-binding modules, which differ in their residue specificity. Canonical histones can be exchanged with histone variants (H2A.Z, H2B.Z, H3.3, CenH3), which differ in amino acid composition from their respective core histone and can carry distinct post-translational modifications. Histone variants play important and distinct roles in *Plasmodium*. Figure adapted from Cui and Miao (2010). (B) Chromatin is assembled as nucleosomes, which comprise ~155 bp DNA wound around the histone octamer. The *P. falciparum* genome is largely maintained in a euchromatic, transcriptionally competent state, while regulated heterochromatin formation is responsible for gene silencing. Post-translational modification of histones affects chromatin architecture through compaction or loosening of the nucleosomes, which in turn controls the accessibility of RNA polymerases and transcription factors to promoters.

parasites also showed a significant downregulation of gametocyte-specific genes (Sinha et al., 2014). Both Kafsack et al. (2014) and Sinha et al. (2014) further demonstrated the in vitro binding of the recombinant AP2-G DNA binding domain (DBD) to a conserved palindromic motif (GxGTAC/GTACxC), previously identified by Campbell et al. (2010). Importantly, the motif is found within the upstream region of gametocyte-specific genes, including *ap2-g* itself, leaving open the possibility that AP2-G binds upstream of its own coding region and self-enhances its transcription (Sinha et al., 2014). The specific interaction between AP2-G and the specific motif was further confirmed in *P. falciparum* using luciferase reporter constructs under the control of three gametocyte promoters (Kafsack et al., 2014).

While AP2-G is unarguably essential for the execution of the gametocytogenesis–specific transcriptional programme in *P. falciparum* and *P. berghei*, there is no evidence suggesting that it is alone sufficient to initiate sexual development. Commitment to developmental pathways is often defined by the likelihood of a TF interacting with a critical promoter (Shiels, 1999) and AP2-G is likely sufficient to induce gametocytogenesis when it is expressed above a threshold level. However, regulators in non-differentiated parasites or additional players might provide further on/off signals or essential 'checkpoints' prior to or after the point at which AP2-G is acting. To assess these possibilities, it is important that the transcriptome is examined at the single cell level. It needs to be determined whether a threshold level of AP2-G is required in

a cell to trigger gametocytogenesis and whether every cell expressing AP2-G develops into a gametocyte or if additional regulators are required. Gametocyte gene development 1 (GDV1) could be one such additional regulator (Eksi et al., 2012) and orthologues are found in most *Plasmodium* spp. examined. *Pfgdv1* has been lost in many laboratory strains and exhibits high allele divergence in field populations (Mobegi et al., 2014). Its perinuclear localization, the fact that it is not deregulated upon *pfap2-g* deletion and that its overexpression leads to increased gametocytogenesis suggest a potentially positive role in transcriptional regulation of gametocytogenesis. Its lack of homology with known regulatory factors and its apparent absence in rodent malaria species is puzzling, however another protein with unique sequence but similar structure could be involved where GDV1 is absent (Eksi et al., 2012).

A second member of the ApiAP2 family, AP2-G2, has been shown to play a complementary but crucial role in regulating the onset of gametocytogenesis in *P. berghei*. PbAP2-G2 was first identified by Sinha et al. (2014) as a player involved in gametocyte maturation acting downstream of AP2-G. Yuda et al. (2015) confirmed this finding and further showed that AP2-G2 does not play a role in sexual commitment or sex determination, but it is essential for development of mature functional gametocytes with developed sex-specific morphologies. Interestingly, disruption of *pbap2-g2* resulted in a global reduction in expression of sexual stage-specific genes, although genome wide identification of AP2-G2 binding sites did not identify those as its targets. As seen with AP2-G, the TF AP2-G2 recognises a specific five-base motif (GTTG (T/C) and its reverse complement (A/G)CAAC) in the promoters of its target genes, which interestingly included genes required for asexual proliferation in erythrocytes. The observation that AP2-G2 is first expressed at the onset of morphological divergence from the asexual ring stage suggested that AP2-G2 induces gene repression in the initial phase of gametocyte development. This results in a block of the transcriptional programme that leads to asexual proliferation; a blockade that accompanies conversion to the sexual stages (Yuda et al., 2015).

Recent data derived from a systematic knockout screen of all ApiAP2 genes in *P. berghei* showed that AP2-G2 is not only essential for gametocyte maturation, but in fact plays a major role as a versatile repressor at multiple different parasite stages. More specifically, erythrocytic stages of *ap2-g2* null parasites were characterised by reduced competitive growth (explaining its apparent effector role downstream of AP2-G) and were accompanied by deregulation of expression of sporozoite, liver and ookinete stage genes (Modrzynska et al., 2017). Significant upregulation of sporozoite and liver stage-specific genes in blood stage cultures as a result of *ap2-g2* deletion, had also been noted by Sinha et al. (2014) and Yuda et al. (2015). Interestingly, upregulation of gametocyte-specific genes was also observed when *ap2-g2* was deleted in an *ap2-g* knockout genetic background, despite the absence of gametocytes (Modrzynska et al., 2017). These recent findings lead to the conclusion that although Sinha et al. (2014) and Yuda et al. (2015) established the involvement of AP2-G2 in gametocyte maturation, AP2-G2 is not necessarily a gametocytogenesis-specific TF. It is rather a global repressor controlling expression of many different stage-specific genes, presumably until the repression levels are overcome by the stage-specific ApiAP2 activator.

3. Epigenetic regulation of AP2-G

Preliminary evidence suggesting the epigenetic regulation of AP2-G was the identification of the *pfap2-g* locus as a heterochromatic 'island' and its localization in the perinuclear periphery (Lopez-Rubio et al., 2009). There is a strong link between gene expression and spatial organization within the nucleus and, more specifically, perinuclear localization is associated with enhanced transcriptional silencing in many eukaryotes, including *P. falciparum* (Schneider and Grosschedl, 2007). Importantly, the *pfap2-g* locus is marked by histone 3 lysine 9 trimethylation (H3K9me3) (Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009), a hallmark histone modification of transcriptionally silenced chromatin (Bannister et al., 2001; Cui et al., 2008). In *P. falciparum*, H3K9me3 is enriched in regions that largely correspond to differentially transcribed virulence gene families and plays a pivotal role in the epigenetic silencing of *var* genes (Lopez-Rubio et al., 2009). Another feature strongly suggesting epigenetic regulation of *ap2-g* is its association with Heterochromatin Protein 1 (HP1), a conserved transcriptional repressor (Flueck et al., 2009). H3K9me3 serves as a docking site for HP1, which once bound can oligomerize and condense nucleosomal arrays (Voss et al., 2014). The AP2-G locus and clonally variant gene families share a strong association with HP1 and the repressive H3K9Me3 mark. This strongly suggested that the variant expression of AP2-G seen among isogenic clones is achieved in a similar manner to variant gene families and relies largely, if not entirely, on epigenetic regulation (Rovira-Graells et al., 2012).

The *P. falciparum* genome encodes a single HP1 protein (PFL1005c) that occupies well-defined loci throughout the genome. Only approximately 5% of the PfHP1-bound loci encode proteins conserved across the genus, which include genes implicated in sexual development and, importantly, *pfap2-g* (Flueck et al., 2009). In fact, nearly 40% of known gametocyte genes are associated with PfHP1-bound heterochromatin (Coleman et al., 2014). In their effort to unravel PfHP1 involvement in singular *var* gene expression, Brancucci et al. (2014) created a PfHP1 knockdown parasite line and observed that depletion of PfHP1 resulted in derepression of 52 out of 60 *var* genes and in a remarkable 25-fold increase in gametocytogenesis in the subsequent cycle. Transcriptional profiling revealed an overall derepression of heterochromatic genes and an upregulation of a number of euchromatic genes, including some associated with early gametocytogenesis (*phistb, msrp1*, *nup116, pfhda1*). Most importantly, *pfap2-g* exhibited significant derepression in schizonts one cycle after PfHP1 depletion (Brancucci et al., 2014). The fact that downregulation of PfHP1 triggers synchronous hyperconversion to gametocytes and the significant drop in H3K9me3-enrichment at the *pfap2-g* locus in PfHP1-depleted parasites demonstrates that commitment to gametocytogenesis is PfHP1-dependent and negatively related to HP1 occupancy of *ap2-g* (Brancucci et al., 2014).

In model organisms, HP1 initiates recruitment of histone methyltransferases (HMTs) and has been shown to bind to several proteins implicated in heterochromatin formation, including histone deacetylases (HDACs) (Schotta et al., 2002; Zhang et al., 2002; Grewal and Jia, 2007). This suggests the involvement of such enzymes in transcriptional regulation of *pfap2-g*. Recently, Coleman et al. (2014) bioinformatically identified a putative class II HDAC, *P. falciparum* histone deacetylase 2 (PfHda2), and showed its perinuclear localization in concentrated foci as well as its spatial overlap with PfHP1. PfHda2 knockdown led to a 3-fold increase in sexual conversion and an increasing defect in parasite proliferation one cycle post knockdown (Coleman et al., 2014). Importantly, PfHda2 knockdown was followed by universal transcriptional derepression of the *var* gene repertoire and a 3% genome-wide differential expression, comprising multigene families undergoing antigenic switching (*Pfmc_2TM*, *rif/stevor*) and gametocytogenesis-associated genes. PfHda2-regulated genes were significantly H3K9me3-enriched and lacked H3K9ac. Among those were >70% of PfHP1-associated gametocyte genes, with *pfap2-g* showing the strongest deregulation (Coleman et al.,

2014). These results demonstrate that PfHda2 is involved in the PfHP1-dependent gametocytogenesis-switching pathway.

It is noteworthy that the magnitude of sexual induction in PfHda2-depleted parasites is significantly lower than the 25-fold increase seen in PfHP1-depleted parasites. This disparity could be attributed to different degradation kinetics or inefficient degradation of PfHda2. In fact, the catalytic nature of enzymes could explain why the effect of HDAC depletion is less pronounced than that of PfHP1, a structural protein, as catalysis of the reaction and sufficient activity can occur even at lower abundance of an enzyme. It could also be attributed to compensation of acetylation by other HDACs and to the different cellular functions of PfHP1 and PfHda2 as a histone reader and a histone modifier, respectively. As a histone reader, PfHP1 protects H3K9me3 marks from demethylase activity and promotes methylation and heterochromatin formation by recruiting HMTs. Thus, upon PfHP1 depletion an abrupt loss of methylation marks and loosening of chromatin is expected, rendering *pfap2-g* widely accessible to histone acetyltransferases (HATs). On the other hand, depletion of PfHda2 would result in inefficient removal of acetyl groups. This event has no striking phenotypic outcome, as the shift to permissive heterochromatin is not solely reliant on HDACs, but also other modifiers. It is dependent on the activity of HATs, which also need to compete for the already H3K9me3/HP1-bound locus. Therefore, PfHP1 depletion largely hampers methylation and facilitates acetylation, whilst PfHda2 depletion results in enhancement of acetylation, but does not necessarily correlate with a decrease in methylation.

4. Model of AP2-G-initiated gametocytogenesis

Over the last decade, observations have suggested that sexual development is the default developmental pathway for *P. falciparum*, and that its constitutive repression permits asexual multiplication within the bloodstream of the human host (Dyer and Day, 2000). The reviewed studies provide evidence strongly supporting this hypothesis and merge to propose a preliminary model for the mechanism regulating the developmental switch that triggers gametocytogenesis (Fig. 2). AP2-G has the central role in the model, as it is the essential TF suggested to be the master regulator of gametocytogenesis. Similar commitment mechanisms

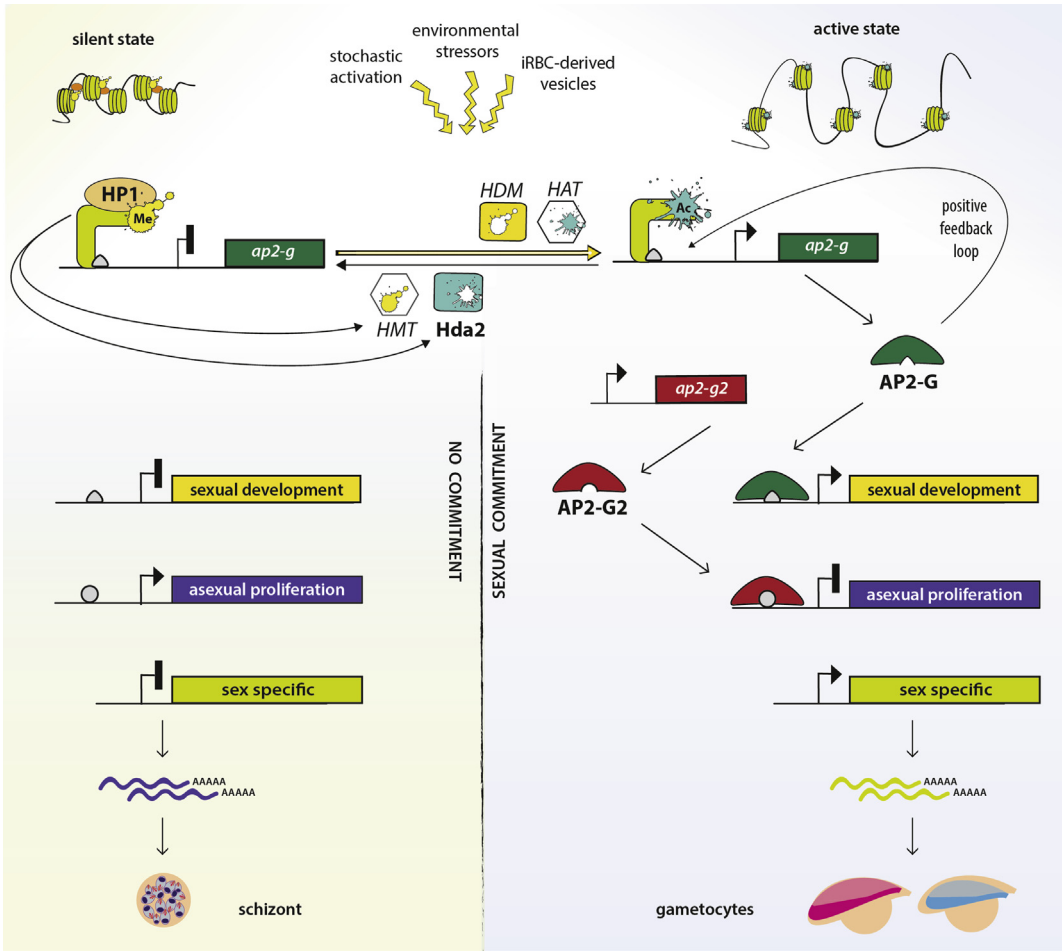

Fig. 2. The proposed mechanism for regulation of sexual commitment in *Plasmodium falciparum*. Sexual commitment requires expression of AP2-G, the key epigenetically regulated transcription factor. The *ap2-g* locus, however, is normally maintained in its silenced state, histone 3 lysine 9 trimethylation-enriched, by ongoing deacetylation and methylation events carried out by histone modifying and binding proteins (yellow panel). Hda2 is involved in deacetylation and an unidentified histone methyltransferase mediates the methylation event. Histone 3 lysine 9 trimethylation (yellow mark) serves as a binding site for Heterochromatin Protein 1, which promotes formation of heterochromatin by further recruiting histone modifying enzymes. Switching from heterochromatin maintenance to the euchromatic, transcription-permissive chromatin state is potentially triggered by environmental signals and is mediated by as yet unidentified histone demethylase and histone acetyltransferase enzymes. When chromatin at the *ap2-g* locus is in its relaxed state, enriched in histone 3 lysine 9 acetylation (turquoise mark), *ap2-g* transcription is permitted (blue panel). The transcription factor is translated and binds to its hexameric motif (grey triangle) found in the promoter region of early gametocyte markers. AP2-G binding to its specific motif upstream of its own coding sequence auto-enhances its transcription and boosts gametocyte inducing signals. This initiates the gametocytogenesis-specific transcriptional programme. In all blood stage parasites AP2-G2 represses genes specific for other life cycle stages (eg. sporozoites/liver stages etc; not shown). In sexually committed parasites AP2-G2 additionally binds to its specific motif (grey circle) upstream of genes required for asexual proliferation and represses their transcription, facilitating conversion to the sexual stages. iRBC, infected red blood cell.

are likely used by other *Plasmodium* spp., as the effector enzymes are conserved across the genus. Moreover, the presence of AP2-G DBD orthologues in other apicomplexans suggests that sexual commitment strategies may also be conserved across the phylum (Sinha et al., 2014).

Transcriptional silencing of *ap2-g* during asexual replication in this model is maintained by removal of acetyl groups from histones mediated to some extent by Hda2 (Coleman et al., 2014). This facilitates H3K9 methylation by HMTs and permits binding of HP1, which recruits further H3K9-specific HMTs and presumably Hda2, in concert with other H3K9-specific HDACs. These orchestrated events lead to formation and propagation of heterochromatin and reduce accessibility of the transcriptional machinery to the promoter and start site of transcription, thus inhibiting *ap2-g* transcription. These events are continual, maintaining silencing of *ap2-g*. Once repression is overcome, transcription and translation of AP2-G take place and the key TF binds to its specific palindromic motif found upstream of its sequence. This ensures its enhanced production and maximal activity of its promoter (Waters, 2016). This model predicts that when the concentration of AP2-G exceeds the critical threshold required for sexual commitment, transcription of downstream gametocytogenesis genes initiates. Alongside the AP2-G triggered gametocytogenesis cascade, AP2-G2 appears to play a key role in repressing the asexual proliferation transcriptional programme as well as genes involved in other lifecycle stages, thereby allowing morphological divergence of sexually committed cells from the asexual blood stages. It is possible that a threshold level of AP2-G would be required to compete with AP2-G2 for the promoters of gametocyte-specific genes, a mechanism which in the absence of AP2-G2 reduces normal levels of commitment and impairs full gametocyte development (Sinha et al., 2014; Modrzynska et al., 2017).

Despite our current understanding of the epigenetic mechanism regulating AP2-G expression and sexual commitment, the processes responsible for triggering the permissive histone state remain elusive. It has long been believed that sexual forms are produced spontaneously and stochastically, which is consistent with the low numbers of non-sequestered, stage V gametocytes observed in parasite populations (Baker, 2010). This also coincides with the low frequency activation of *pfap2-g*. Observations, however, suggest that sexual commitment is also sensitive to environmental stimuli. Host immune cells and hormones, products of haemolysis and certain anti-malarial drugs are some of the many external factors known to increase gametocytogenesis (Smalley and Brown, 1981; Schneweis et al., 1991; Escobedo et al., 2005; Peatey et al., 2009). Gametocyte conversion rates throughout infection also vary with resource availability and the presence of other parasite genotypes. Notably, the parasite population genetic make-up and haematological factors not only influence gametocyte conversion rates, but also shape the relative investment in male and female gametocytes (Reece et al., 2008, 2005). The extent to which parasites respond directly to external signals or to the effect those signals have on the population state is unknown (Cameron et al., 2013). Crucially, the epigenetic control suggested in this model would permit flexible control of gametocyte induction that can be responsive to environmental signals.

The beauty of epigenetic control is its plasticity; it can facilitate responses to both stochastic and environmental influences. The fact that chromatin-modifying enzymes are susceptible to environmental fluctuations (Turner, 2009), suggests that triggering the permissive state for commitment by environmental signals is a possibility. The conserved and repeated occurrence of the AP2-G binding motif upstream of the *ap2-g* locus implies that AP2-G likely enhances activity of its own coding region via a positive autoregulatory feedback loop, amplifying inducing signals in response to external stimuli and self-sustaining its expression until the 'threshold' level required for commitment is reached. Fine-tuning transcriptional regulation by feedback control is often seen in unicellular organisms when a quick and precise response to changing environmental conditions is needed, as exemplified by the prototypical mating response in *Saccharomyces cerevisiae* (Cook et al., 1996; Tedford et al., 1997; Bardwell et al., 1998; Zeitlinger et al., 2003). A positive feedback loop would facilitate rapid transition to the gametocytogenesis-specific transcriptional programme, providing quick adaptation to fast-fluctuating and diverse environments. An example of an environment to which parasites may respond to by switching to sexual stages is the bone marrow, where significant enrichment of immature *P. falciparum* gametocytes has been noted (Aguilar et al., 2014; Joice et al., 2014). When parasites reach the bone marrow, gametocytogenesis may be induced by high concentrations of soluble factors such as those found in erythrocyte progenitors (Trager et al., 1999; Peatey et al., 2013), or even by an insufficiency of specific metabolites required for asexual growth. There is considerable evidence to support the bone marrow as a site of specific adaption to which parasites respond by inducing gametocytogenesis, but the possibility that gametocytes also develop elsewhere and simply lodge in the bone marrow cannot currently be excluded (Rogers et al., 2000).

Sensing of external signals requires the operation of signalling pathways. Sequencing and biochemical data support the existence of signalling features in *Plasmodium* and there is evidence of pathway-mediated environmental sensing in related apicomplexans (Dixon et al., 2008; Hartmann et al., 2013). For instance, cAMP-mediated stress signalling is important for trachyzoite-to-bradyzoite development in *Toxoplasma gondii* (Hartmann et al., 2013). Involvement of a protein kinase activated by cAMP-dependent and phorbol-ester-induced pathways has been proposed in sexual commitment, but no upstream effector molecules have been identified (Dyer and Day, 2000; Baker, 2010). Interestingly, treatment of parasite cultures with cholera toxin, which ADP-ribosylates the α-subunit of heterotrimeric G-protein receptors, induces gametocytogenesis, suggesting involvement of G-protein coupled receptors in sexual triggering (Dyer and Day, 2000; Peatey et al., 2013). The molecular factors likely to trigger such pathways and subsequent sexual conversion remain elusive, but two recent studies reported that cell–cell communication with infected RBC-derived exosomes/microvesicles increases sexual conversion rates in vivo and in vitro (Mantel et al., 2013; Regev-Rudzki et al., 2013). Hence, an appealing scenario, consistent with a quorum sensing mechanism, is that these vesicles and/or the cargo they deliver trigger a signalling cascade, which in a density-dependent manner results in the eviction of PfHP1 and *pfap2-g* transcription.

5. Unidentified players and key questions to be answered

Despite the recent progress in revealing major events in the molecular mechanism of commitment, many questions remain unanswered. The proposed model mechanism only encompasses the basic skeleton of proteins currently known to regulate sexual commitment. However, additional events and players are likely involved. For instance, histone demethylases (HDMs) and HATs are expected to act in concert with HP1 and PfHda2 in this pathway, but remain unidentified. *Plasmodium falciparum* has two predicted Jumonji-C domain (JmjC) containing HDMs, which are enzymes that demethylate tri-methylated histone residues in model organisms, PfJmjC1 and PfJmjC2 (Duffy et al., 2012). PfJmjC2 stands as the likely demethylase candidate for the proposed model as it is present in all *Plasmodium* lineages, in contrast to PfJmjC1,

orthologues of which have been found in only three *Plasmodium* spp. so far (Cui et al., 2008). The *P. falciparum* genome contains eight putative HATs, including the conserved PfGCN5, the orthologue of which acetylates H3K9 in yeast (Fan et al., 2004). Analysis by Cui et al. (2007) revealed a genome-wide co-distribution of PfGCN5 and H3K9ac, suggesting its involvement in the molecular pathway of commitment.

While the HP1-recruited enzyme mediating the methylation event in the proposed mechanism remains unidentified, it is established that the *P. falciparum* genome encodes 10 lysine HMTs, all of which belong to the SET domain family (Duffy et al., 2012). It is unknown how H3K9 trimethylation is established in *Plasmodium*, but PfKMT1 (also known as PfSET3), which is conserved throughout the genus, is a potential candidate (Cui et al., 2008). It is the homologue of the well-characterised KMT1, which methylates H3K9 in *Drosophila melanogaster* (known as Su(var)3–9) (Greil et al., 2003) and *Schizosaccharomyces pombe* (known as Cir4) (Allis et al., 2007). PfKMT1/PfSET3 showed a clustered distribution in the heterochromatic nuclear periphery and its partial overlap with H3K9me3, CenH3 and the nuclear envelope, strengthens its predicted role (Lopez-Rubio et al., 2009; Volz et al., 2010). Previous attempts to delete PfKMT1/PfSET3 were lethal for the parasite (Lopez-Rubio et al., 2009; Jiang et al., 2013), therefore inducible gene knockdown systems will be necessary to gain insight into its function and investigate its involvement in sexual commitment. PfKMT1/PfSET3 is a very strong HMT candidate and obtaining experimental evidence of its involvement could provide valuable insight into epigenetic control of sexual commitment.

The presence of a complex involved in epigenetic control of sexual commitment could also be investigated, based on the interaction of HP1 and class II HDACs in mammalian cells and with KMT1 in *Drosophila* (Greil et al., 2003). Other proteins, such as PfGDV1, could also be involved in this complex or with other mediating factors acting upstream of AP2-G. The perinuclear localization of PfGDV1 (suggesting a role as a transcriptional regulator), the fact that its overexpression leads to increased gametocytogenesis and the observation that its expression is not deregulated by *pfap2-g* deletion, suggests it is not acting downstream in the AP2-G-initiated cascade (Eksi et al., 2012). Thus, the likelihood that it acts upstream of *ap2-g,* potentially in concert with the identified histone modifiers, should be examined.

Transcriptomic profiling of the *ap2-g* knockout and PfHP1 and PfHda2 knockdown lines has provided the scientific community with an inventory of genes encoding prospective downstream molecular players. Brancucci et al. (2014) identified genes transcribed in the early phase of gametocytogenesis in PfHP1-depleted parasites, which encode proteins involved in host cell remodelling. Transcriptomic analysis of PfHda2-depleted parasites revealed a number of differentially expressed genes that could be downstream targets of PfAP2-G and some heterochromatin-marked early-gametocytogenesis genes (*Pfge2, Pfge7, Pfge8*) (Coleman et al., 2014), which could be regulated by histone modifying enzymes. A further meta analysis of all then available transcriptome data, supplemented by new transcriptome data derived from in vivo samples, integrated these data to define modules of genes including, amongst others, clusters upregulated around sexual commitment (Pelle et al., 2015). The identification of the genes directly regulated by PfAP2-G will require a dedicated PfAP2-G genome-wide ChIP analysis.

The discovery of AP2-G provides the potential to experimentally regulate sexual commitment, a tool that would be revolutionary in deciphering the upstream signalling and downstream gene regulation pathways leading to and following AP2-G activation. To understand how temporal and responsive gametocytogenesis is achieved, the factors triggering the euchromatic state and the contribution of signal transduction pathways in this process should be identified. This is a missing aspect in the understanding of epigenetic regulation of *ap2-g* expression. Comparative analyses in other *Plasmodium* spp., such as *P. berghei*, will be important to identify the conserved features in this process and distinguish those from features seen only in *P. falciparum*, as it has been demonstrated that early genes lying downstream of PfGDV1 are species-specific (Eksi et al., 2012) and most likely involved in the extreme remodelling of the host erythrocyte.

6. Conclusions

The studies discussed in this review have unravelled some of the earliest known events and molecular players involved in parasite transmission. Research from Kafsack et al. (2014) and Sinha et al. (2014) has unequivocally demonstrated that AP2-G is the essential TF initiating sexual differentiation in *P. falciparum* and *P. berghei*. Brancucci et al. (2014) and Coleman et al. (2014) reinforced this finding and showed that PfAP2-G is epigenetically regulated by reversible chromatin formation, mediated by PfHP1 and PfHda2, processes that are likely to be conserved. Work by Yuda et al. (2015) identified AP2-G2 as a transcriptional repressor essential for gametocyte maturation and Modrzynska et al. (2017) revealed its major role as a universal repressor. The model mechanism for sexual commitment derived from the reviewed findings would allow a basal level of low frequency gametocyte investment, the rate of which is determined by the genetic makeup of the parasite. Importantly, however, it implies that loss of the ability to produce gametocytes in mechanical passaging and parasite culture could be a result of epigenetic phenomena and chromatin organization rather than DNA mutations only, which has been the assumption to date.

While commitment to gametocytogenesis is undoubtedly regulated by coordinated changes in the structure of chromatin, the prospect that it is subject to several layers of control is probable. However, their nature remains an open question. Therefore, the contribution of types of epigenetic control, other than histone modifications, such as nucleosome occupancy and the three dimensional (3D) nuclear organization in space, could be evaluated (Ay et al., 2015). Additionally, mapping the 4D view of chromatin, the spatiotemporal nuclear architecture and the transcriptional landscape in relation to the consequent phenotype, will provide a novel view on regulation of gene expression, insight into the potential multi-layered control of this and other developmental pathways and could also inform about antigenic variation (Sekelja et al., 2016). Finally, the potential that AP2-G and AP2-G2 are regulated at the post-transcriptional level cannot be excluded. Regulation of localization, stability and the rate of translation of mRNAs plays a role in developmental processes in eukaryotes and there is evidence of RNA-binding proteins (PfPuf2, Pfg27) regulating sexual development in *P. falciparum* (Sharma et al., 2003; Miao et al., 2010). The switch to sexual development could be driven not only by the levels of AP2-G present, as determined by its level of expression, but also by the levels of active protein present. This could be achieved by means of post-translational regulation, such as changes in protein conformation, protein stability and post-translational modifications, for example phosphorylation.

The described model of commitment would allow a variable baseline production of gametocytes, further adjusted in response to environmental stressors for optimal infection-transmission outcome. The intriguing resemblance between epigenetic regulation of *pfap2-g* and the *var* gene family, raises the prospect that commitment to gametocytogenesis is one form of bet-hedging, as seen in the distinct examples of expression of PfEMP1 antigens and intraerythrocytic adaptation. An isogenic population 'hedges its bets' with stochastic switches between the active and repressed

states of *ap2-g* to cover all grounds; intra-host survival and onward transmission, ensuring its survival in the next experienced environment and its success at a population level (Rovira-Graells et al., 2012). Bet-hedging could be one explanation for the heterogeneous decision to commit to sexual development, but it is unlikely to be the only one as commitment to gametocytogenesis seems to also be responsive to environmental influences. Detailed investigation of the mechanism underlying commitment, its direct comparison to the one underlying antigenic variation of PfEMP1 and identification of the potential sensing apparatus and signalling molecules involved in environmental sensing would address these questions.

An interesting hypothesis made by Kafsack and colleagues (2014) is that *pfap2-g* expression is (at least in part) regulated by the same mechanism that ensures singular expression of *var* genes, implying that *pfap2-g* expression could be mutually exclusive with expression of specific *var* gene subsets. The hypothesis is based on the similar silencing fashion seen in *pfap2-g* and *var* genes and the fact that insulator-like pairing elements (PEs) required for maintenance of *var* gene silencing also flank the *pfap2-g* locus (Kafsack et al., 2014). Whether the PEs play a similar role in *pfap2-g* silencing could also be investigated. Importantly, sharing this mechanism would enable sexually committed parasites to 'hide' specific PfEMP1 antigens during the 8–12 days of the gametocyte development period, thus protecting transmission stages from clearance by the host immune recognition mounted against asexual stages. The co-regulation of *pfap2-g* and *var* genes is an appealing hypothesis, however singular expression of *var* genes is mediated by PfSET2 and PfSET10, which methylate H3K36 and H3K4, respectively, (Lopez-Rubio et al., 2009, 2007; Volz et al., 2012), while the *pfap2-g* locus is largely devoid of these marks (Lopez-Rubio et al., 2009). Furthermore, PfSET2 is absent in rodent malaria species and its knockout had no effect on expression of gametocyte genes (Jiang et al., 2013). Nevertheless, the arena is certainly worth investigating, as even a partial mutual regulation of these transcriptional programmes would mechanistically link parasite transmissibility and virulence, having significant implications in the context of disease control.

These studies not only provided the first model for sexual commitment in *Plasmodium*, but also gave invaluable information for the discovery of transmission-blocking antimalarial agents. AP2-G is a plausible drug target candidate due to its conservation throughout the phylum and its lack of orthologues in humans, a factor that is common to all ApiAP2 proteins. The homodimerization of certain *Plasmodium* AP2 domains, which is likely required for their function, implies that agents disrupting this process could be developed as transmission-blocking agents (Campbell et al., 2010). Notably, if a shared mechanism regulates virulence mechanisms and gametocytogenesis, targeting one or two processes could shift the balance towards infectiousness or transmissibility, with dramatic effects at a population level. Targeting upstream regulatory players would avoid disrupting this equilibrium and would target both processes. Such targets include the histone modifying enzymes which evidently have important regulatory roles in malaria parasite biology. As HDACs have already been evaluated as promising chemotherapeutic targets and HDAC inhibitors have been used to cure rodent malaria in the past (Mai et al., 2004), targeting of PfHda2 could be considered. Moreover, two diaminoquinazoline-based compounds, a class of HMT inhibitors, have shown promising activity against both asexual and sexual stages of multiple *Plasmodium* spp. They exhibit a rapid-killing profile and display oral efficacy in in vivo mouse models (Malmquist et al., 2015). Furthermore, parasiticidal activity of HAT inhibitors has also been reported (Cui et al., 2007).

These findings also provide the malaria research community with new practical tools. Inducing gametocytogenesis in vitro has previously required application of environmental stress and achieved 10–20% sexual conversion (Silvestrini et al., 2005). Discovery of the gametocytogenesis on/off switch could enable experimentally controlled sexual induction and batch culturing of gametocytes by genetic manipulation. This could facilitate work on sexual stages, could provide a more controlled experimental set-up and could allow the development of screening assays. Furthermore, the lack of markers for sexually committed schizonts and rings has been a long-standing hurdle. The transcriptional data obtained by such studies could provide us with numerous gene candidates that could be used as very early markers, which is invaluable for field studies too.

Substantial effort has been put into studying *Plasmodium* intraerythrocytic stages and similar efforts should now be put into the obstruction of sexual stages, as a reduction in transmission would allow a large leap forward in malaria control. The reviewed studies have greatly contributed to the long-standing goal of deciphering sexual differentiation in *Plasmodium* and the proposed commitment model for *P. falciparum* provides a basis for unravelling the epigenetic and molecular pathways regulating this developmental switch. The reviewed findings strengthen our understanding of developmental transitions in *Plasmodium* and lay the groundwork for stimulating virulence, evolutionary and developmental studies in *Plasmodium* and related pathogens.

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