VECTOR-BORNE DISEASES

Sexual development in *Plasmodium* parasites: knowing when it's time to commit

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Abstract | Malaria is a devastating infectious disease that is caused by blood-borne apicomplexan parasites of the genus *Plasmodium*. These pathogens have a complex lifecycle, which includes development in the anopheline mosquito vector and in the liver and red blood cells of mammalian hosts, a process which takes days to weeks, depending on the *Plasmodium* species. Productive transmission between the mammalian host and the mosquito requires transitioning between asexual and sexual forms of the parasite. Blood-stage parasites replicate cyclically and are mostly asexual, although a small fraction of these convert into male and female sexual forms (gametocytes) in each reproductive cycle. Despite many years of investigation, the molecular processes that elicit sexual differentiation have remained largely unknown. In this Review, we highlight several important recent discoveries that have identified epigenetic factors and specific transcriptional regulators of gametocyte commitment and development, providing crucial insights into this obligate cellular differentiation process.

Trophozite
A highly metabolically active asexual form of the malaria parasite that forms during the intra-erythrocytic developmental cycle following the ring stage.

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Malaria affects almost 200 million people worldwide and causes 584,000 deaths annually[1]; thus, developing a better understanding of the mechanisms that drive the development of the transmissible form of the malaria parasite is a matter of urgency. The disease is caused by unicellular protists of the *Plasmodium* genus, of which five species are known to infect humans (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*). The most virulent of these is *P. falciparum*, owing to its ability to cytoadhere to the microvasculature and evade clearance by the immune system[2]. Following infection, which is initiated by the bite of a mosquito vector, the parasite exits the skin, develops in the liver of the human host and is then released into the vasculature to infect red blood cells (FIG. 1). During the blood stage of infection, malaria parasites undergo repeated cycles of red blood cell invasion, replication, egress and reinvasion, which results in all of the clinical manifestations of the disease, including the characteristic periodic spikes of fever. *P. falciparum* displays a 48-hour red blood cell replicative cycle, during which the parasite progresses through ring, trophozoite and schizont stages[3]. The ring-stage parasite is the earliest form of the parasite that is established following invasion of the red blood cell by a merozoite; when stained with Giemsa and viewed under the microscope, it resembles a flat disc. After the ring stage, the parasite rounds up as it enters the trophozoite stage, in which it is far more metabolically active and expresses surface antigens for cytoadhesion. The trophozoite grows substantially in size to prepare for the subsequent schizont stage, which is characterized by asexual replication and cell division (schizogony), ultimately resulting in the production of 16–32 merozoites[3]. The merozoites are released into the bloodstream when the red blood cell ruptures, and the released merozoites invade new red blood cells, thereby initiating the next wave of blood-stage development.

The next major stage of the parasite life cycle involves transmission to the mosquito vector. Asexual blood-stage parasites cannot productively infect the mosquito; transmission of parasites from the human host to the mosquito requires sexual differentiation in the blood to form male and female gametocytes. This process (which is known as gametocytogenesis) occurs in only a small proportion of blood-stage parasites both *in vivo* and *in vitro*[4], but is an essential step in the *Plasmodium* spp. life cycle, as it ensures continued transmission. Following ingestion by the mosquito, gametocytes experience a drop in temperature, a change in pH and exposure to xanthurenic acid, which together trigger their maturation

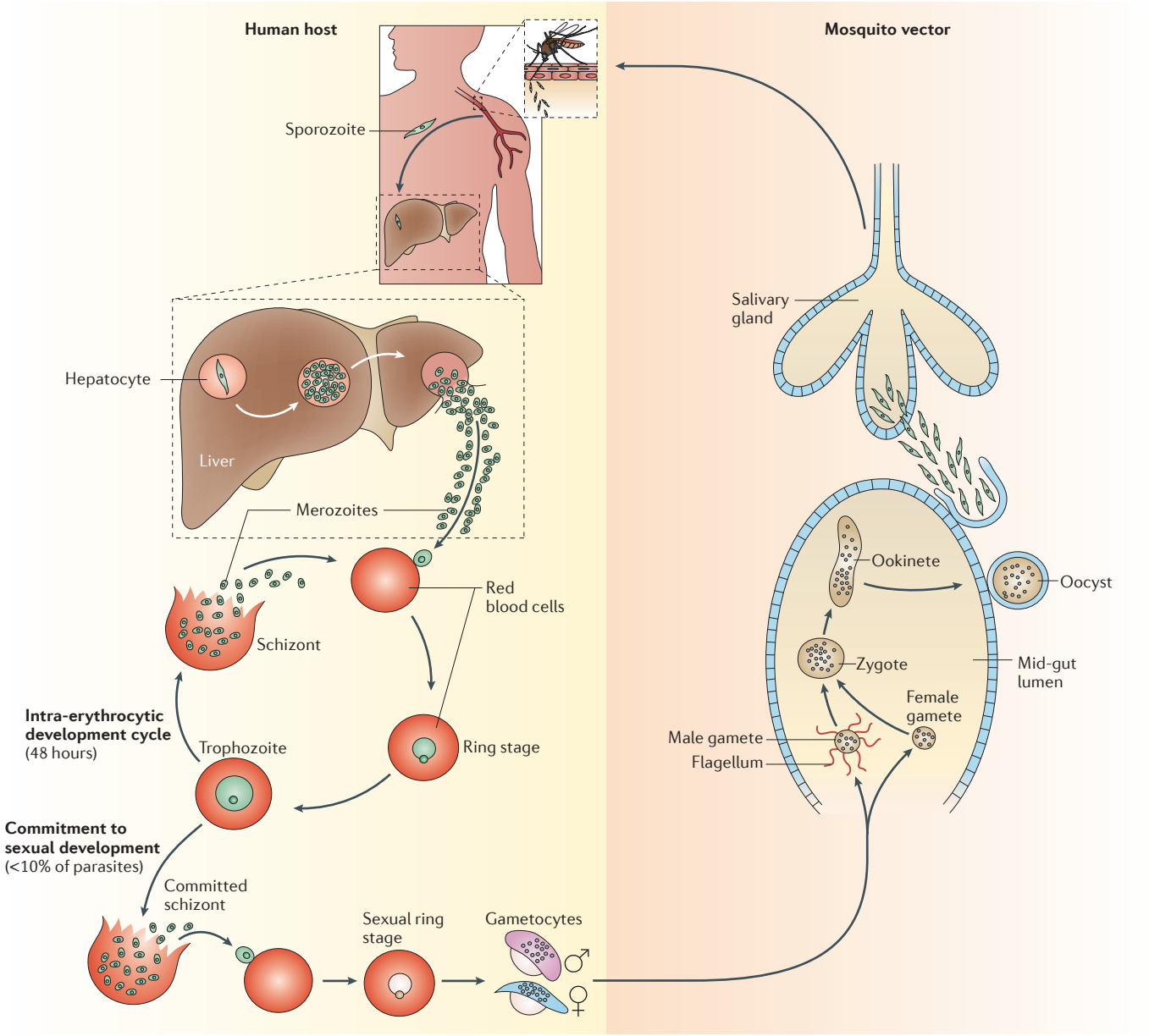

Figure 1 | **The life cycle of *Plasmodium falciparum*.** Sporozoites are transmitted to the human host via the bite of an infected *Anopheles* spp. mosquito and then travel to the liver. Following development in the liver, tens of thousands of merozoites are released into the blood and invade red blood cells. The parasites then undergo repeated rounds of asexual multiplication (the intra-erythrocytic developmental cycle), progressing through ring, trophozoite and schizont stages in turn. In each cycle, a small proportion (<10%) of parasites instead begin to develop into the sexual form of the parasite, which is known as a gametocyte. This form is required for productive transmission to the mosquito host. Commitment to sexual development is thought to occur in schizonts, which form sexual rings and then gametocytes following reinvasion of red blood cells. After 10–12 days of development, mature male and female gametocytes are taken up by the mosquito, in which they undergo the sexual phase of the life cycle. Gametocytes differentiate into gametes and, after fertilization, the resulting zygote (which develops into a motile form known as the ookinete) forms an oocyst. When the oocyst ruptures, haploid sporozoites are released, migrate to the salivary glands and can then be transmitted to humans.

into gametes in the mosquito mid-gut[5] (FIG. 1). Male gametocytes rapidly undergo three rounds of DNA replication to form an octoploid nucleus, followed by the assembly of eight flagella to produce sexually competent male gametes, which are released in a process known as exflagellation. This remarkable transformation occurs in less than 20 minutes and is among the fastest known DNA-replication events in eukaryotes[6]. Haploid male gametes then fuse with female gametes to form a diploid zygote, which develops into an ookinete in which meiotic recombination occurs[7]. Ookinetes traverse the mid-gut epithelial cell wall and form oocysts, which

Schizont
A multinucleated, asexual form of the malaria parasite that forms during the intra-erythrocytic developmental cycle after several rounds of replication. Mature schizonts contain many merozoites, which are released when the red blood cell ruptures.

Ring-stage parasite
An asexual form of the malaria parasite that forms very soon after invasion of the red blood cell by a merozoite during the intra-erythrocytic developmental cycle.

Merozoite
Cell released either from intra-erythrocytic schizonts or from infected hepatocytes that can invade red blood cells.

Schizogony
The process by which many merozoites are produced through asexual reproduction.

Xanthurenic acid
A metabolite found in the mosquito mid-gut that induces gametogenesis in *Plasmodium spp.*.

Exflagellation
The process by which flagellated male gametes are produced and released from a male gametocyte (for *Plasmodium* spp., in the mosquito mid-gut).

Oocysts
Structures formed by ookinetes (for *Plasmodium* spp., in the mosquito mid-gut) from which sporozoites are released.

Sporozoites
Cells that are released from oocysts. *Plasmodium* spp. sporozoites reside in the salivary glands of the mosquito. These are released into the bloodstream of the human host on infection and then invade liver cells.

Haemocoel
The body cavity of the mosquito.

Glucose-6-phosphate dehydrogenase
An enzyme involved in the pentose phosphate pathway. In humans, genetic deficiency of this enzyme leads to an increased risk of haemolysis, particularly in response to certain triggers.

ultimately rupture to release haploid sporozoites that actively migrate through the haemocoel to the salivary glands, where they reside until the mosquito takes a blood meal, which simultaneously delivers sporozoites to the next human host[8].

Gametocytogenesis is a crucial step in the *P. falciparum* life cycle, as it is required for parasite transmission from the human host to the mosquito and thus for the subsequent infection of other humans. Given the importance of gametocyte transmission and the increased focus on the eradication of malaria, new therapeutic agents are urgently required to combat sexual-stage parasites. Most antimalarial drugs in use today, including chloroquine and the artemisinin-combination therapies recommended by the WHO for front-line treatment, are not as effective against mature gametocytes as they are against asexual-stage parasites [9–11]. Therefore, even though the parasite forms that are responsible for causing the symptoms of malaria are killed, gametocytes can persist for over a month and thus can still be transmitted to mosquitoes[12]. The only currently approved antimalarial drug that is highly effective against gametocytes is primaquine, which unfortunately can cause haemolysis in individuals with glucose-6-phosphate dehydrogenase deficiency[13]. Gaining a better understanding of the molecular pathways that trigger asexual parasites to commit to sexual development and of the early events in the commitment process is fundamental to the development of intervention strategies that prevent malaria transmission and to achieving the goal of malaria eradication[14]. Although gametocytogenesis has been the subject of several comprehensive reviews[15–20], the molecular mechanisms governing commitment have been elusive until recently. In this Review, we focus primarily on recent studies that have shed light on the role of transcriptional and epigenetic regulation during gametocyte commitment, and we contextualize these findings with regard to the existing body of work in this exciting area of research.

Commitment to sexual development

Commitment to sexual development occurs at some point before schizogony, as all merozoites within a mature schizont will either differentiate into gametocytes or continue asexual development[21]. However, the precise timing of commitment and the triggers involved are currently unclear[22–24]. In general, parasite exposure to different environmental stressors, such as high host parasitaemia or drug treatment, is associated with an increase in the rate of commitment *in vitro*. This makes evolutionary sense for the parasite: if the host is dying (for example, as a result of high parasitaemia) then transmission to the mosquito must occur if the parasite is to survive. However, reproductive restraint also has a role in determining whether commitment occurs, as the parasite faces a trade-off between continued asexual proliferation and transmission to a new host[25,26]. Indeed, in natural infections, drug treatment and high parasitaemia do not necessarily lead to increased numbers of gametocytes[27,28]. Reported triggers of gametocyte induction *in vitro* include factors such as high parasitaemia, use of conditioned medium (growth medium that has already been used to culture cells and is thus nutrient depleted) and treatment with the antimalarial drug chloroquine[29–32]. Interestingly, extracellular vesicles released by infected red blood cells have recently been implicated as factors in conditioned medium that contribute to this increase[33,34]. However, the component or components within vesicles that function as triggers remain undefined. Attempts have been made to optimize the conditions for inducing gametocyte development by exploiting known triggers of gametocytogenesis, but even then only a minority of cells commit to producing gametocytes[35,36]. The low frequency of conversion *in vitro* and *in vivo* suggests that, although environmental factors seem to influence the probability of conversion for a particular cell, the decision to commit is essentially stochastic.

When gametocytogenesis has been triggered, subsequent gametocyte development in *P. falciparum* is a lengthy process that lasts approximately 10–12 days, which is substantially longer than in other *Plasmodium* species, such as the rodent parasite *Plasmodium berghei*, in which gametocytogenesis takes slightly longer than a day. The morphology of *P. falciparum* gametocytes is also different[37], further suggesting that the regulation of gametocytogenesis is likely to have diverged between species. Owing to the differences between gametocyte development in *P. falciparum* and in other malaria-causing species, here we focus mainly on gametocytogenesis in *P. falciparum*, except for relevant aspects when more is known about other *Plasmodium* species.

Gametocyte development in *P. falciparum* is traditionally divided into five stages (stages I–V) that can be distinguished microscopically[29], and molecular markers for various stages of development have been reported[38] (FIG. 2). Whereas stage I gametocytes resemble asexual trophozoites, in subsequent stages gametocytes begin to acquire a characteristically crescent or sickle shape, from which the name 'falciparum' (from the Latin '*falx*' (sickle) and '*parere*' (to give birth)) is derived. For most of their development, the majority of gametocytes are sequestered in the bone marrow, where they are protected from clearance by the spleen, so only early (stage I) and late-stage mature gametocytes (stage V) are readily detectable in the peripheral blood[39–41].

Despite the essentiality of sexual development *in vivo*, loss of gametocytogenesis *in vitro* owing to spontaneous gene deletions is relatively common, with several parasite lines having been reported that either are unable to form gametocytes or produce greatly reduced numbers[42–47]. Among these, the F12 gametocyte-non-producing line (a clone of the 3D7 parasite line) has been extensively characterized[42,48,49]. Gametocyte-non-producing lines probably arise because *in vitro* parasites are not required to undergo sexual development, so there is no selective pressure to retain functional versions of the genes required for this process. Indeed, as commitment to gametocytogenesis precludes cells from continuing to multiply asexually, parasites unable to commit would be expected to have a selective fitness advantage. This has been directly demonstrated recently both through the

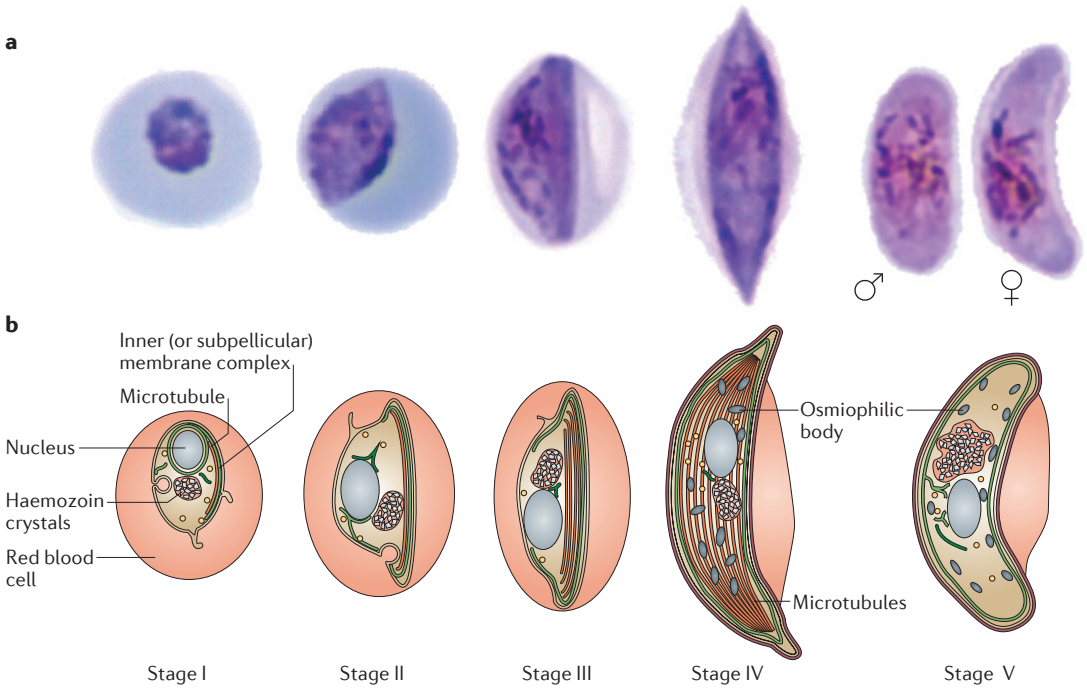

Figure 2 | **The stages of gametocyte development in *Plasmodium falciparum*.** Development of a mature *Plasmodium falciparum* gametocyte occurs over 10–12 days and is traditionally divided into five morphologically distinct stages (shown as photographs (part **a**) and schematics (part **b**)). Stage I gametocytes are indistinguishable from asexual trophozoites, but stage II gametocytes are readily discernible as they begin to elongate; the parasites become D-shaped and the subpellicular microtubules begin to form. In stage III, gametocytes elongate further and the ends become rounded. In females, mitochondria and Golgi bodies proliferate (not shown). Stage IV gametocytes continue to elongate but now have pointed ends, and osmiophilic bodies and extensive rough endoplasmic reticulum (not shown) develop in females. Gametocytes eventually assume their characteristically crescent shape in stage V. In this stage, male and female gametocytes can be readily differentiated, as the females are more elongated and curved than the thicker males. Using a Giemsa stain, stage V females stain blue and males stain pink. Images in part **a** are reproduced with permission from Sharp, S. 2007. Ph.D. thesis. University of London, London, United Kingdom. Part **b** is adapted with permission from REF. 151, Elsevier.

generation of gametocyte non-producers by mechanical passage and from head-to-head *in vitro* growth competition with gametocyte-producing and gametocyte-non-producing parasites, in which the non-producing line quickly outcompetes the other[47,50].

Genes that affect gametocytogenesis

Identifying genes that are important for regulating the early stages of commitment and gametocyte development has proved difficult, and few genes have been identified as being essential for gametocytogenesis. Genes that have been shown to have a functional role in gametocytogenesis and their possible functions (where known) are summarized in TABLE 1. Some genes that affect the ratio of male to female gametocytes have also been identified, suggesting that they have a role in sex-specific gametocyte development (BOX 1). One of the earliest-identified genetic alterations associated with loss of gametocytogenesis *in vitro* is a deletion on chromosome 9, and at least two genes in this region have been implicated in this phenotype[42,43]. Targeted disruption of *pfgig* (*P. falciparum* gene implicated in gametocytogenesis, accession numbers (from PlasmoDB) PF3D7_0935600/PFI1720w — for clarity, current and former accession numbers for genes are provided throughout) leads to a reduction in the number of gametocytes, and complementation of the gene results in increased expression of the early gametocyte gene *pfs16* (PF3D7_0406200/PFD0310w)[51]. The function of the protein encoded by *pfgig* is unknown. The second gene in the same region of chromosome 9 that has been implicated in the regulation of gametocytogenesis is *pfgdv1* (*P. falciparum* gametocyte development 1, PF3D7_0935400/PFI1710w)[52]. *pfgdv1* was identified as a gene associated with gametocytogenesis by comparing the genomes of two parasite lines derived from the same parental strain that differ in their ability to form gametocytes; the parasite line that is unable to form gametocytes has a deletion of the *pfgdv1* locus, and complementation with the full-length gene restores its ability to form gametocytes. Deletion of *pfgdv1* is also associated with reduced expression of known gametocyte genes, as well as of other genes that may be early markers of gametocytogenesis. Intriguingly, immunofluorescence assays show that PfGDV1 localizes to the periphery of the nucleus, suggesting that it may be involved in gene regulation. Several other genes in this deleted region encode proteins that have increased expression in gametocytes compared to asexual parasites[53], indicating that they could also have a role in gametocytogenesis, although these have not yet been investigated further.

Extracellular vesicles
Small vesicles that are released by cells from all three domains of life; in malaria infections, extracellular vesicles are released by infected red blood cells and are thought to have a role in cell–cell communication.

Recently, transposon-mediated mutagenesis has been used to screen for gametocyte non-producers in an effort to determine the full repertoire of genes required for gametocyte development[54]. In total, 16 genes were identified using this approach, many of which were not previously implicated in gametocytogenesis. Some of the mutants are unable to form early stage gametocytes, whereas other mutants remain able to do so but are blocked later in gametocyte development. Complementation of five of these genes (*P. falciparum* HSC70-interacting protein (*pfhip*; PF3D7_0527500/PFE1370w), *pfmaf1* (PF3D7_0416500/PFD0800c), gametocyte erythrocyte cytosolic protein (*pfgeco*; PF3D7_1253000/PFL2550w), splicing factor 3A subunit 3 (*pfsf3a3*; PF3D7_0924700/PFI1215w) and PF3D7_0532600/PFE1615c) was attempted, and for four of them (all but *pfgeco*) the ability to form gametocytes was restored. This suggests that these genes do have a specific role in some stage of gametocyte development. However, two of the genes identified in this study, *pfgeco* and *P. falciparum* LCCL domain-containing protein 2 (*pfccp2*; PF3D7_1455800/PF14_0532) have been successfully disrupted previously in other studies with no effect on gametocytogenesis, despite their expression in gametocytes[55,56]. The remaining genes identified thus require further validation to confirm whether they are in fact required for gametocyte development.

Other genes have been shown to have a role in sexual development using a more targeted approach to disrupt genes known to be expressed in gametocytes. For example, *pfs16* is one of the earliest known markers of gametocytogenesis, and the protein it encodes is found on the parasitophorous vacuole membrane[57]. Although its function is unknown, genetic ablation of *pfs16* leads to reduced gametocytaemia[58]. Another well-established early gametocyte marker, *P. falciparum* gamete antigen 27/25 (*pfg27*; PF3D7_1302100/PF13_0011), was reported to be essential for gametocytogenesis, but subsequent work has shown that this is not the case[59,60]. Pfg27 may be involved in RNA metabolism, as it is present in the nucleus, it interacts with RNA *in vitro* and it has been shown to form a complex with predicted RNA-binding proteins[61,62]. Finally, recent work has shown that disruption of the gene encoding the ATP-binding cassette (ABC) transporter P. *falciparum* ABC sub-family G member 2 (*pfgABCG2*; PF3D7_1426500/PF14_0244), which is involved in lipid metabolism, leads to an increase in the number of gametocytes[63].

Gene expression during gametocytogenesis

Another important approach to explore the molecular mechanisms that drive commitment has been to investigate transcriptional changes that occur in sexually committed cells. To better define these transcriptional differences, the ideal approach would be to compare gene expression profiles between committed and non-committed parasites. Unfortunately, this is extremely difficult owing to the low proportion of committed cells (generally below 10%) and the paucity of markers of committed gametocytes that would enable these cells to be isolated at the earliest stages of commitment. Owing to these obstacles, studying pure populations of committed cells has not been possible, so the field has turned to characterizing differences between gametocyte-producing and gametocyte-non-producing lines[46,48,49,52]. One study found that a cluster of 119 genes are downregulated in the gametocyte-non-producing F12 parasite line compared with in a gametocyte-producing 3D7 clone[49]. This gene set includes *pfs16* and *pfg27,* which are known to be expressed in early gametocytes[57,64], and the stage-specificity of several of the novel predicted gametocyte genes was validated by northern blot and immunofluorescence assays[49]. One of these, *pfpeg4* (also known as *etramp10.3*; PF3D7_1016900/PF10_0164), is also expressed in a subset of schizonts and may be a marker for committed parasites[65]. Similarly, a comparison of the transcriptomes of two 3D7 clones obtained through limiting dilution cloning identified several genes that were highly expressed in the gametocyte-producing clone compared with a gametocyte-deficient clone[46,52]. Most strikingly, several genes belonging to a family of subtelomeric genes located on chromosome 14 were identified. Although most of these genes are primarily expressed in stage I and stage II gametocytes, two of them (*pfg14.744* (PF3D7_1477300/PF14_0744) and *pfg14.748* (PF3D7_1477700/PF14_0748)) are also expressed in a small proportion of schizonts. These schizonts are potentially those that have committed to sexual development, suggesting that these two genes are expressed in committed cells and are thus among the earliest known genes to be expressed during gametocytogenesis[65]. For this reason, *pfg14.748* has been used as a marker for early gametocytes[39,41]. Proteomic analysis of F12 and 3D7 parasites has confirmed that the proteins encoded by these genes are expressed in early gametocytes but not in asexual parasites, and many known gametocyte-specific proteins (including Pfg27 and Pfs16) were also identified in the same analysis[53]. Interestingly, many of the gametocyte-specific proteins identified (such as Pfg14.744 and Pfg14.748) are predicted to be exported from the parasite, which suggests that remodelling of the host red blood cell is important for early gametocyte development, but the function of these proteins and their potential roles in remodelling have not been investigated.

Other work has focused on gene expression in purified gametocytes to define the transcriptomic profiles of gametocytes at different stages of maturation[66–68]. Differential display analysis has been used to compare the transcriptomes of mature gametocytes with those of asexual parasites, identifying 80 genes expressed in gametocytes but not in asexual-stage parasites[67]. Similarly, microarray analysis of various gametocyte stages and of asexual-stage parasites identified a subset of 246 genes that are expressed in sexual-stage parasites only[66]. This analysis led to the identification of two motifs (TGTANNTACA and TCCTT) that are enriched in the upstream regions of genes involved in sexual conversion. More recently, RNA sequencing has been used to characterize the transcriptomes of parasites at various stages, including stage II and stage V gametocytes[68]. In addition to the identification of several gametocyte-specific

Parasitophorous vacuole
A vacuole that surrounds an intracellular parasite (in the case of the malaria parasite, within the red blood cell).

Gametocytaemia
The fraction of red blood cells that contain gametocytes.

Limiting dilution cloning
A method used to obtain parasite lines derived from a single parasite. The parental cell line is heavily diluted and grown in individual wells so that on average no more than one parasite is present per well.

Subtelomeric genes
Genes located in close proximity to the telomere.

Differential display analysis
A PCR-based method for identifying differentially expressed genes between two samples.

Table 1 | **Genes that functional evidence shows have a role in regulating *Plasmodium falciparum* gametocytogenesis**

Accession number*	Gene name and/or description	Effect of disruption on gametocyte production	Effect of complementation and/or overexpression on gametocyte production	Putative function
PF3D7_1302100/ PF13_0011	Gamete antigen 27/25 (Pfg27)	50–100-fold decrease (disruption)[45]; no effect on numbers, but many have abnormal morphology (disruption)[44]	ND	RNA metabolism[61,62]
PF3D7_0406200/ PFD0310w	Pfs16	4-fold decrease (disruption)[58]	ND	Unknown
PF3D7_0935600/ PFI1720w	Gene implicated in gametocytogenesis (Pfgig)	6-fold decrease (disruption)[51]	Upregulation of *pfs16* (complementation)[51]	Unknown
PF3D7_1216500/ PFL0795c	Male gametocyte development 1 (Pfmdv-1; also known as Pfpeg3)	20-fold decrease; fewer male gametocytes (disruption)[121]	ND	Unknown
PF3D7_0417100/ PFL0795c	mRNA-binding protein PfPuf2	2-fold increase, increased male gametocytes (truncation)[96]	Restoration to wild-type gametocyte levels (complementation); decrease in gametocytes (overexpression)[96]	Translational repression[92,96]
PF3D7_0518700/ PFE0935c	mRNA-binding protein PfPuf1	Decrease (disruption)[15]	ND	Translational repression[91]
PF3D7_0935400/ PFI1710w	Gametocyte development 1 (PfGDV1)	Decrease (naturally occurring deletion)[52]	Restoration to wild-type gametocyte levels (complementation); increase in gametocytes (overexpression)[52]	Unknown
PF3D7_1343000/ MAL13P1.214	Phosphoethanolamine *N*-methyltransferase (PfPMT)	5-fold decrease and arrest at stage III gametocytes, resulting in a lack of mature gametocytes (disruption)[152]	Restoration to wild-type gametocyte levels (complementation)	Lipid metabolism[152]
PF3D7_1455800/ PF14_0532	LCCL domain-containing protein 2 (PfCCp2)	No gametocytes (*piggyBac* transposon insertion into ORF)[49]; no effect (disruption)[56]	ND	Unknown
PF3D7_0527500/ PFE1370w	HSC70-interacting protein (PfHIP)	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	ND	Heat-shock protein co-chaperone[153]
PF3D7_0304400/ PFC0200w	60S ribosomal protein L44, putative	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	Restoration to wild-type gametocyte levels (complementation)[54]	A ribosomal subunit; functions in translation
PF3D7_0416500/ PFD0800c	Repressor of RNA polymerase III transcription MAF1, putative	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	Restoration to wild-type gametocyte levels (complementation)[54]	Represses RNA polymerase III
PF3D7_1409900/ PF14_0097	Cytidine diphosphate-diacyl-glycerol synthase (PfCDS)	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	ND	Phospholipid synthesis[154]
PF3D7_1236600/ PFL1770c	p25α family protein, putative	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	ND	Unknown
PF3D7_1115900/ PF11_0167	DHHC-type zinc-finger protein, putative	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	ND	Unknown
PF3D7_1253000/ PFL2550w	Gametocyte erythrocyte cytosolic protein (PfGECO)	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]; no effect (disruption)[50]	Did not restore wild-type gametocyte levels (complementation)[54]	Heat-shock protein[55]
PF3D7_1317200/ PF13_0097	Transcription factor with AP2 domain(s)	No gametocytes (*piggyBac* transposon insertion into ORF)[54]	ND	Transcription factor[75]
PF3D7_0924700/ PFI1215w	Splicing factor 3A subunit 3 (SF3A3), putative	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	Restoration to wild-type gametocyte levels (complementation)[54]	RNA metabolism
PF3D7_0532600/ PFE1615c	*Plasmodium* exported protein, unknown function	No gametocytes (*piggyBac* transposon insertion into ORF)[54]	Restoration to wild-type gametocyte levels (complementation)[54]	Unknown
PF3D7_0730900/ MAL7P1.171	*Plasmodium* exported protein, unknown function	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	ND	Unknown
PF3D7_0920000/ PFI0980w	Long chain fatty acid elongation enzyme, putative	No gametocytes (*piggyBac* transposon insertion into ORF)[54]	ND	Lipid metabolism
PF3D7_0714100/ PF07_0055	Conserved *Plasmodium* protein, unknown function	No gametocytes (*piggyBac* transposon insertion 5′ of ORF)[54]	ND	Unknown

Table 1 (cont.) | **Genes that functional evidence shows have a role in regulating *Plasmodium falciparum* gametocytogenesis**

Accession number*	Gene name and/or description	Effect of disruption on gametocyte production	Effect of complementation and/or overexpression on gametocyte production	Putative function
PF3D7_1462500/ PF14_0595	Conserved *Plasmodium* protein, unknown function	No gametocytes (*piggyBac* transposon insertion into ORF)[54]	ND	Unknown
PF3D7_1222600/ PFL1085w	PfAP2-G	No gametocytes (disruption and FKBP destabilization domain§)[50]	ND	Transcription factor[50,75]
PF3D7_1220900/ PFL1005c	Heterochromatin protein 1 (PfHP1)	25-fold increase (FKBP destabilization domain§)[67]	No effect reported[65]	Epigenetic regulation[78,79]
PF3D7_1008000/ PF10_0078	Histone deacetylase 2 (PfHda2)	3-fold increased (FKBP destabilization domain‡)[80]	ND	Epigenetic regulation[80]
PF3D7_1426500/ PF14_0244	ATP-binding cassette sub-family G member 2 (PfgABCG2)	2-fold increase[63]	ND	Lipid metabolism[63]

FKBP, FK506-binding protein; ND, not determined; Pf, *Plasmodium falciparum*. *Accession numbers are from PlasmoDB. Both the current and former accession numbers for each gene are listed. ‡The FKBP destabilization domain system is used for inducible protein knockdown.

genes (particularly those expressed in stage V gametocytes), many antisense transcripts were found, which suggests that additional layers of regulation may have a role in gametocyte formation. Although RNAi is thought to be absent from *Plasmodium* spp., non-coding RNAs have been implicated in transcriptional regulation[69].

Transcriptional regulation of gametocytogenesis

Despite the identification of candidate genes involved in gametocytogenesis and transcriptional changes occurring in committed cells, the key drivers of sexual differentiation have not been identified through transcription-based approaches. Recent work has taken advantage of the relative ease of whole-genome next-generation sequencing to identify genomic, rather than transcriptional, changes in gametocyte-non-producing parasite lines and has identified single nucleotide polymorphisms in genes encoding proteins potentially involved in gametocyte commitment. Using this approach, the transcription factor AP2-G was recently identified as a master regulator of gametocytogenesis in both *P. falciparum* and *P. berghei* (PF3D7_1222600/ PFL1085w and PBANKA_143750, respectively)[47,50] (FIG. 3). AP2-G belongs to the apicomplexan-specific family of AP2 DNA-binding proteins (known as the ApiAP2 family) (BOX 2), which are characterized by the presence of APETALA2/ethylene response factor (AP2/ERF) DNA-binding domains[70]. The ApiAP2 family is considered to be the major family of transcription factors in *Plasmodium* spp., as other transcription factors have not been identified to date. Previously, other ApiAP2 proteins have also been shown to be important in developmental stage transitions of the malaria parasite, including oocyst-, sporozoite- and liver-stage formation[71–73].

In *P. falciparum*, mutations in *pfap2-g* were initially identified in two independent gametocyte-non-producing lines, including the well-studied F12 3D7 clone, providing an explanation for the inability of this line to produce gametocytes. As expected, deleterious mutations, such as those in *pfap2-g,* are not found in field isolates, as wild parasites need to be transmission competent[50]. Consistent with this, *pfap2-g* expression is substantially higher in field isolates compared with laboratory-adapted parasite strains[74]. Targeted disruption of the *ap2-g* locus results in the complete loss of gametocytogenesis in both *P. falciparum* and *P. berghei,* and results in the downregulation of many genes that are usually expressed in early gametocytes[47,50]. In agreement with this observation, the DNA motif (GTAC) to which the AP2 domain of AP2-G binds is located upstream of many known gametocyte genes (such as *pfs16, pfg27* and *pfg14.744*), and gel shift assays have confirmed that PfAP2-G binds to the promoters of several gametocyte genes[50,75]. In addition, PfAP2-G was shown to drive expression of a reporter gene regulated by promoters containing this motif, whereas expression was blocked when the motif was mutated[50]. Cumulatively, these

Box 1 | **Sex determination**

Plasmodium falciparum does not have sex chromosomes, and very little is known about the mechanism by which parasite sex is determined. However, it has been shown that all merozoites derived from a single committed schizont become either male or female, and there is never a mixture, indicating that sex determination occurs soon after, or simultaneously with, commitment[23,120]. Furthermore, more female than male gametocytes are produced; as each male gametocyte produces eight gametes, whereas each female gametocyte produces a single gamete, this does not necessarily lead to more female gametes being produced than male gametes. The study of sex determination remains greatly unexplored, and very few genes that affect sex ratio have been identified. The Dd2 parasite line produces a greatly reduced number of male gametocytes, and genetic analysis has revealed that this is due to low levels of *P. falciparum* male gametocyte development 1 (*pfmdv1*) transcripts in gametocytes[44,121]. Disruption of *pfmdv1* (PF3D7_1216500/PFL0795c) leads to the arrest of gametocyte development at stage I and an extremely low ratio of male to female gametocytes. PfMDV-1 localizes to membranes, such as the plasma membrane and parasitophorous vacuole membrane, so it is unlikely to have a role in gene regulation. Another gene that potentially contributes to the deficiency of male gametocytes in Dd2 parasites was also found on chromosome 14 but has not yet been investigated further[121]. Disruption of the RNA-binding protein PfPuf2 also skews sex ratios in *P. falciparum*, in addition to leading to an increase in gametocytaemia[96]. In wild-type parasites, the sex ratio at the onset of gametocytogenesis is biased towards male gametocytes and later shifts to a female bias, whereas in parasites in which PfPuf2 has been disrupted more male gametocytes are produced throughout gametocyte development. Further upstream regulators of sex determination in gametocytes remain to be identified.

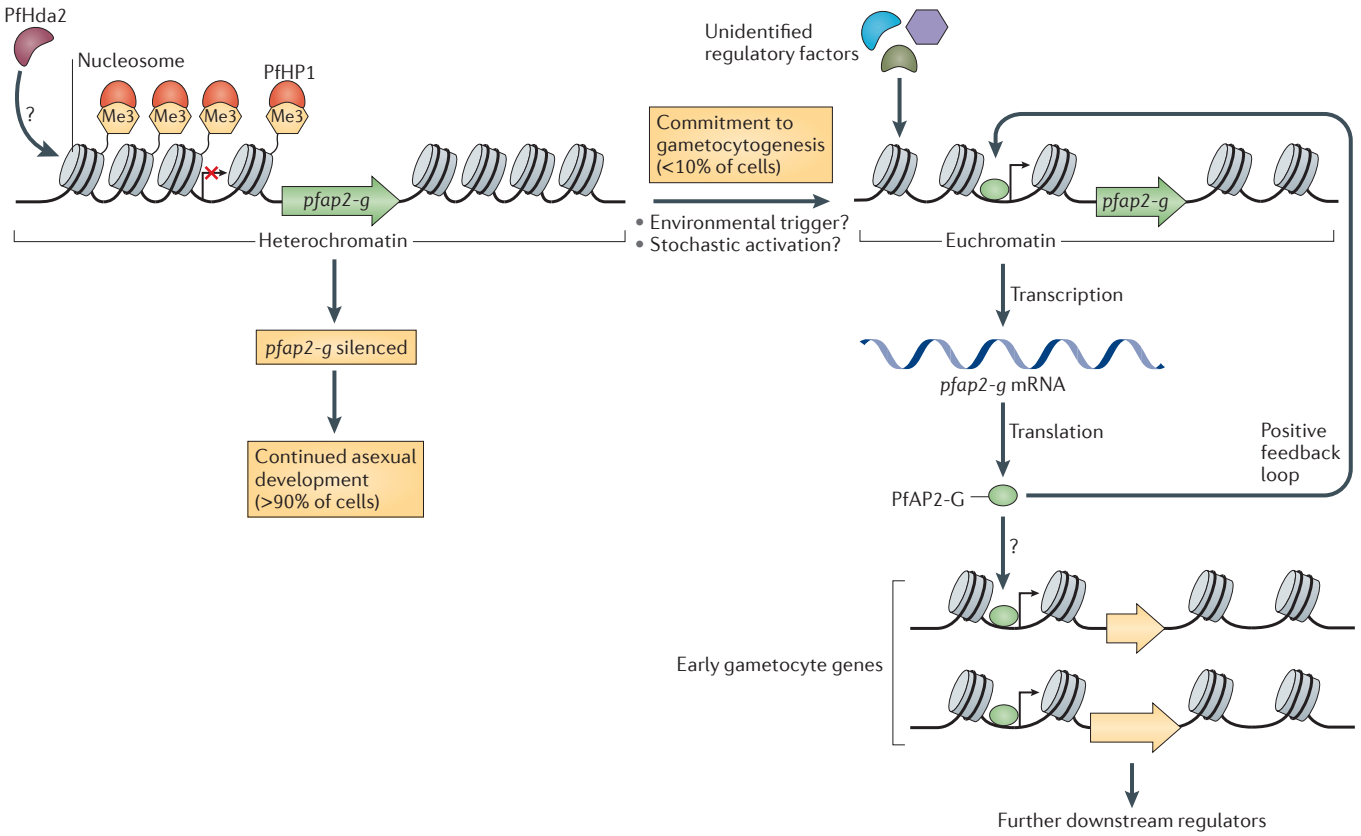

Figure 3 | **Proposed model for the regulation of sexual commitment in *Plasmodium falciparum*.** In a population of *Plasmodium falciparum*-infected red blood cells, *pfap2-g* (which encodes the master regulator of gametocytogenesis) is transcribed in only a small proportion of parasites (less than 10%). The fraction of cells expressing *pfap2-g* correlates with those that will become gametocytes, suggesting that these cells are committed to gametocytogenesis. In the majority of cells, the *pfap2-g* locus is heterochromatic and enriched in histone H3 trimethylated on lysine 9 (H3K9me3)[98]. H3K9me3 is bound by *P. falciparum* heterochromatin protein 1(PfHP1), which silences *pfap2-g* expression[78,79,146]. *P. falciparum* histone deacetylase 2 (PfHda2) is also required for silencing of the *pfap2-g* locus, although the mechanism involved is unclear[80]. In a small proportion of cells, H3K9me3 is removed and the *pfap2-g* locus becomes euchromatic, resulting in *pfap2-g* expression. Although the exact events that occur remain unclear, one possibility is that *pfap2-g* is expressed in a stochastic manner in individual cells and that certain environmental signals can increase the rate of activation of the *pfap2-g* locus. As yet unidentified transcription factors, histone-modifying enzymes and chromatin-remodelling complexes are also likely to be involved in this process. A rise in PfAP2-G levels probably activates the expression of early gametocyte genes, thereby initiating gametocytogenesis. Expression of *pfap2-g* itself is autoregulated, as PfAP2-G binds to its own promoter. Although most early gametocyte genes are euchromatic, several are heterochromatic (not shown) and thus are also negatively regulated by PfHP1 and PfHda2; it is unclear whether PfAP2-G directly regulates these genes or whether PfAP2-G and other heterochromatic early gametocyte genes share a common activator. Other transcriptional regulators, such as PfAP2-G2 (not shown), may be involved in regulating gametocytogenesis downstream of PfAP2-G, although this remains to be shown experimentally.

data strongly suggest that AP2-G induces the expression of genes involved in early gametocytogenesis and thus may drive commitment. In both *P. falciparum* and *P. berghei*, the *ap2-g* locus contains several copies of its own motif in its promoter, which suggests that it potentially regulates expression of itself in a positive-feedback loop[47,50]. Consistent with this, deletion of these motifs in *P. berghei* results in an almost complete loss of gametocyte formation[47]. Importantly, the percentage of cells expressing PfAP2-G correlates linearly with the percentage of cells that form gametocytes, indicating that PfAP2-G-expressing cells are committed to form gametocytes. Interestingly, parasites in which *pfap2-g* has been disrupted have a growth advantage over wild-type parasites[50]. These data suggest that PfAP2-G is required for parasite commitment to gametocytogenesis, and parasites in which *pfap2-g* has been disrupted have a growth advantage probably because these parasites do not divert resources away from asexual development for sexual differentiation. However, whether PfAP2-G is the factor that drives commitment to gametocytogenesis or whether it is instead activated immediately after commitment remains to be determined.

Two other ApiAP2 proteins have also recently been implicated in regulating gametocyte development. In *P. berghei*, disruption of *pbap2-g2* (PBANKA_103430) leads to a severe reduction in the number of gametocytes, although a small number of female gametocytes

Box 2 | **ApiAP2 DNA-binding proteins**

The apicomplexan AP2 (ApiAP2) DNA-binding proteins were first described in 2005 as a family of putative transcription factors in *Plasmodium* spp. and other Apicomplexa[70]. Each member of this protein family contains a highly conserved 60-amino-acid-long AP2 DNA-binding domain, which is homologous to the APETALA2/ethylene response factor (AP2/ERF) DNA-binding proteins that are ubiquitous in plants[122,123]. *Plasmodium* species contain between 25 (in the rodent parasites *Plasmodium berghei*, *Plasmodium chabaudi and Plasmodium yoelii*) and 27 ApiAP2 proteins, some of which have been characterized in detail[124]. Interestingly, there is one ApiAP2 protein that is unique to *Plasmodium vivax* and *Plasmodium knowlesi*, suggesting the regulation of specialized processes in these divergent species. Similarly to their plant counterparts, ApiAP2 proteins function as transcriptional regulators throughout all stages of parasite development. To date, seven ApiAP2 proteins have been identified as regulators of stage-specific development, including liver-stage maturation (AP2-L)[71], salivary gland sporozoite formation (AP2-Sp)[73], ookinete development (AP2-O)[72] and gametocyte commitment (AP2-G)[47,50] in *Plasmodium* spp., as well as bradyzoite development (AP2IX-9 and AP2XI-4)[125,126], virulence and host invasion (AP2XI-5) in the related apicomplexan parasite *Toxoplasma gondii*[127]. Many ApiAP2 proteins are also predicted to be involved in the regulation of the parasite cell cycle[75,128–131].

Thus far, ApiAP2 proteins seem to have diverse roles in the regulation of transcription. Although many ApiAP2 proteins are thought to function as transcriptional activators, the *T. gondii* protein AP2IX-9 has been demonstrated to function as a repressor of transcription, leading to the inhibition of bradyzoite development[126]. Similarly, there are exceptions in *Plasmodium* spp. as well, such as *Plasmodium falciparum* SPE2-interacting protein (PfSIP2; PF3D7_0604100/PFF0200c), which has been implicated in the establishment of subtelomeric heterochromatin[132]. Interestingly, another ApiAP2 protein (PF3D7_1107800/PF11_0091) is potentially involved in regulating mutually exclusive expression of the *var* multigene family by binding to a sequence within the *var* gene intron and mediating its movement to a region located in the nuclear periphery that is associated with gene activation, probably by recruiting actin[133]. Unsurprisingly, there is evidence from immunoprecipitation experiments, yeast two-hybrid assays and computationally predicted interaction networks that many ApiAP2s may form complexes with other proteins (such as histone-modifying enzymes, histone-binding proteins, predicted chromatin-remodelling factors and other ApiAP2 proteins)[134–136]. Given the large size of many ApiAP2 proteins, interaction with other regulatory proteins is likely to emerge as a common theme for ApiAP2 protein functions in transcriptional regulation.

Although the function of many ApiAP2 proteins remains to be determined, the binding specificities for many of the AP2 domains from *P. falciparum*[75], *T. gondii*[126] and *Cryptosporidium parvum*[131] have been determined *in vitro* using protein-binding microarrays, capturing a diverse repertoire of DNA-binding interactions for this protein family. Although these sequences enable the prediction of target genes regulated by specific ApiAP2 factors, further experimental work using chromatin immunoprecipitation followed by sequencing will be required to specifically determine the regulatory network for each of these proteins. Given the paucity of other characterized sequence-specific transcription factors, ApiAP2 proteins are widely viewed as the major transcriptional regulators that work together with post-transcriptional control mechanisms (such as translational repression and mRNA stability[137]) and epigenetic regulation[138] to govern parasite development.

are still produced[47]. Surprisingly, microarray analysis has revealed that disruption of *pbap2-g2* leads to the upregulation of genes normally expressed in sporozoites and liver-stage parasites, indicating that it may function as a repressor of these genes. It is possible that derepression of such genes leads to aberrant gametocyte development owing to the expression of these genes at the wrong stage of development (O. Billker and K. Modrzynska, personal communication). The *P. falciparum* orthologue of PbAP2-G2 remains uncharacterized. Finally, a third ApiAP2 protein, PF3D7_1317200/PF13_0097, was identified by transposon mutagenesis; insertion of the transposon into the coding sequence of the gene resulted in the inhibition of gametocyte production[54]. Gel shift assays have shown that the AP2 domain of PF3D7_1317200 binds to the promoters of several gametocyte genes only if its motif is present, providing further support for its role in the regulation of gametocytogenesis (M.L., unpublished observations). However, owing to a missense mutation in *pfap2-g* in the parasite line containing a transposon in PF3D7_1317200, it is unclear whether the gametocyte-deficient phenotype is due to disruption of PF3D7_1317200 or to mutation of *pfap2-g* (M.L., unpublished observations). Disruption of *pfap2-g* does not affect expression of PF3D7_1317200, suggesting that if PF3D7_1317200 is also involved in gametocytogenesis it may be upstream of PfAP2-G[50]. However, the role of PF3D7_1317200 and the hierarchy of gene expression during commitment and gametocyte development remain to be verified experimentally.

Epigenetic regulation of gametocytogenesis

One of the most intriguing observations regarding AP2-G and the regulation of sexual differentiation is that epigenetic control of *pfap2-g* is crucial for this process in *P. falciparum*. Epigenetic regulation has long been known to be important for transcriptional control in *P. falciparum*, particularly in the regulation of clonally variant gene families (such as the *var*, repetitive interspersed family (*rif*), *P. falciparum* Maurer's cleft 2 transmembrane (*pfmc-2tm*) and subtelomeric variable open reading frame (*stevor*) gene families)[76] (BOX 3). One indication that *pfap2-g* may be regulated epigenetically arose from work examining transcriptional differences between isogenic parasite lines[77], which found that *pfap2-g* expression varies between clones, consistent with epigenetic regulation. It has also been known for some time that the *pfap2-g* locus is enriched in the silencing factor *P. falciparum* heterochromatin protein 1 (PfHP1; PF3D7_1220900/PFL1005c). HP1 is a highly conserved protein in eukaryotes that broadly regulates gene expression, but in *P. falciparum* it has an unusually restricted distribution and is associated primarily with the clonally variant gene families, as well as with a small number of other genes, including *pfap2-g*[67,68]. As in other organisms, PfHP1 binds to histone H3 trimethylated on lysine 9 (H3K9me3), a histone mark generally associated with transcriptional repression. Remarkably, depletion of PfHP1 leads to the derepression of *pfap2-g* and a dramatic increase in gametocyte production[78,79]. Depletion of PfHP1 also leads to upregulation of many known early gametocyte genes that are not associated with PfHP1; these genes may be directly regulated by PfAP2-G, although further work is required to establish this. Several other early gametocyte genes (including *pfg14.744*, *pfg14.748* and others in the subtelomeric regions of chromosome 14) are also enriched in PfHP1, so it is possible that derepression of these genes contributes to the increased numbers of gametocytes observed[46,79]. Interestingly, PfHP1 is only required in schizont-stage parasites to repress gametocytogenesis, suggesting that PfAP2-G might be required only in schizonts to initiate gametocyte development[78].

Chromatin immunoprecipitation followed by sequencing
A genome-wide approach that uses next-generation sequencing to identify DNA sequences directly or indirectly bound by a protein (such as a transcription factor or modified histone).

Box 3 | **Epigenetic regulation of antigenic variation**

Much of the work on gene regulation in *Plasmodium falciparum* has focused on the regulation of the *var* multigene family. The *var* gene family comprises approximately 60 members that encode variants of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates the cytoadhesion of *P. falciparum* parasites to the vascular endothelium[139,140]. PfEMP1 is trafficked to the red blood cell surface, where it enables the parasites to sequester in host tissues and evade clearance in the spleen, resulting in enhanced virulence[2]. The regulation of PfEMP1 production is also crucial in the evasion of the host immune system through a process known as antigenic variation[141]. In each parasitized red blood cell, only one *var* gene is expressed at a time, thereby presenting a single type of PfEMP1 molecule to the host immune system[142]. Owing to the large repertoire of *var* genes, the parasite can, in theory, evade the host's immune system indefinitely by switching the expression of these genes, thus maintaining a chronic infection. This mutually exclusive expression of *var* genes is regulated at the level of transcription[143].

Epigenetic regulation is crucial for *var* gene regulation[144]. Epigenetic regulation refers to heritable changes in gene expression that result from changes in chromatin structure rather than alterations in DNA sequence[145]. Silenced genes are tightly packaged with histone proteins to form heterochromatin, whereas the more open form of chromatin, euchromatin, is more transcriptionally permissive. Heterochromatin in *P. falciparum* has a very restricted distribution that is mostly limited to clonally variant gene families, such as the *var* genes[79,146]. However, the single *var* gene that is active is euchromatic[143,146,147].

Although the *var* genes are the best-studied clonally variant gene family, there are several others that are thought to be regulated epigenetically[77]. These include the repetitive interspersed family (*rif*), subtelomeric variable open reading frame (*stevor*) and *P. falciparum* Maurer's cleft 2 transmembrane (*pfmc-2tm*) multigene families, which also encode surface antigens. Mutually exclusive expression has not been demonstrated for any of these families, but they do show some similarities with *var* gene regulation[148,149]. Epigenetic regulation of genes involved in red blood cell invasion and in solute transport has also been demonstrated[150], showing that epigenetic mechanisms are probably important in regulating a wide range of processes in the parasite.

Another epigenetic factor, *P. falciparum* histone deacetylase 2 (PfHda2; PF3D7_1008000/PF10_0078), has also recently been shown to be involved in negatively regulating gametocytogenesis[80]. As seen for depletion of PfHP1, depletion of PfHda2 leads to the derepression of *pfap2-g*, upregulation of known early gametocyte genes and an increase in the number of gametocytes produced. However, these effects are less dramatic in the case of PfHda2 depletion compared with that of PfHP1 depletion. PfHda2 is thought to function upstream of PfHP1, as its depletion specifically perturbs the expression of those genes that are bound by PfHP1. One possibility is that histone deacetylation at these loci by PfHda2 is required before methylation of H3K9 and subsequent binding of PfHP1, resulting in the formation of silenced heterochromatin. These observations are consistent with the hypothesis that in large populations of malaria-infected red blood cells *ap2-g* is epigenetically silenced in the majority of cells, and that this silencing is relieved in those cells destined to become gametocytes. However, despite the correlation between AP2-G expression and commitment at the population level, it has yet to be demonstrated that individual cells expressing AP2-G are committed to become gametocytes.

Although many other proteins predicted to be associated with chromatin have been identified in proteomic studies of *P. falciparum* gametocytes[53], the only one apart from PfHP1 and PfHda2 to be investigated in gametocytes is *P. falciparum* nucleosome assembly protein (PfNAPS; PF3D7_0919000/PFI0930c, originally called PfSET). Although PfNAPS is also transcribed in asexual-stage parasites, the gene has two stage-specific promoters[81]. One promoter is active only in asexual parasites, whereas the other is only active in a small number of schizonts (potentially those that are committed to gametocytogenesis) and in male gametocytes. Although PfNAPS has been shown to interact with histones in asexual stage parasites, its function in gametocytes has not been examined[82,83]. Thus, the role of PfNAPS and those of other chromatin-associated proteins expressed in gametocytes remain to be elucidated.

Post-transcriptional regulation

In addition to transcriptional and epigenetic regulation, post-transcriptional regulation in *Plasmodium* spp. parasites has a key role in the regulation of gametocyte commitment and subsequent sexual development, and has been recently reviewed[84]. Several *Plasmodium* spp. genes have been identified that are highly transcribed in gametocytes but are not translated, suggesting that they are subject to translational repression[85]. The proteins *P. berghei* development of zygote inhibited (PbDOZI; PBANKA_121770) and *P. berghei* homolog of worm CAR-I and fly trailer hitch (PbCITH; PBANKA_130130) are important in translational repression in *P. berghei* gametocytes, although their orthologues have not been extensively characterized in *P. falciparum*, except for evidence showing that PfDOZI is expressed in asexual stage parasites and *in vitro* data showing that it can bind RNA and inhibit translation[86]. In *P. berghei,* PbDOZI and PbCITH are required for zygote development but not for gametocytogenesis[87,88]. RNA immunoprecipitation experiments in *P. berghei* identified 731 transcripts (representing half of all transcripts present in gametocytes) associated with either PbDOZI or PbCITH, including nearly all of those that were found to be downregulated in response to deletion of PbDOZI[89]. That so many transcripts were found to be associated with PbDOZI and PbCITH highlights the importance of translational repression in sexual development.

In addition to DOZI and CITH, *Plasmodium* spp. parasites encode hundreds of other RNA-binding proteins that may also be involved in regulating gene expression through similar mechanisms[90]. Of these, only the Puf (Pumilio- and FEM-3-binding factor) family of RNA-binding proteins has received substantial attention to date. *P. falciparum* encodes two Puf proteins, both of which are expressed in gametocytes and bind to mRNA[91–93]. PfPuf1 (PF3D7_0518700/PFE0935c) has been reported to be involved in gametocytogenesis as deletion of *pfpuf1* results in a decrease in the number of gametocytes produced by *P. falciparum*[15]. By contrast, *pbpuf1* (PBANKA_123350) deletion does not affect gametocyte development[94,95], highlighting important molecular differences in gametocytogenesis between the human and rodent parasites. Disruption of *pfpuf2* (PF3D7_0417100/PFL0795c) resulting in expression of a truncated form of the transcript leads to an increase in gametocytaemia, as well as to major changes in the abundance of stage III and stage V gametocyte transcripts[92,96].

Euchromatin
Loosely packaged chromatin that is transcriptionally permissive — this is the predominant state of the chromatin in *Plasmodium falciparum*.

Heterochromatin
Tightly packaged chromatin that is associated with silencing marks such as histone H3 trimethylated on lysine 9 (H3K9me3). Genes in these regions are generally silenced.

Chromatin
DNA packaged with proteins (particularly histones, but also other proteins).

Expression of 13% of the *P. falciparum* transcriptome is altered in response to *pfpuf2* disruption[96], including an increase in many of the genes that have been shown to be translationally repressed in *Plasmodium* spp. gametocytes, suggesting that this protein is responsible for translational repression of these genes. In blood-stage cultures of PfPuf2-knockout parasites, *pfap2-g* transcripts are substantially elevated, which may explain the increase in gametocytaemia, although this remains to be verified[92,96] (M.L., unpublished observations). Unlike most other organisms (in which Puf proteins bind to only the 3′-untranslated regions of target genes), the Puf-binding elements in *Plasmodium* spp. are found in both the 5′-untranslated region and the 3′-untranslated region[92]. There are conflicting reports regarding the effect of disruption of PbPuf2 (PBANKA_071920) in *P. berghei*; one study reported an increase in gametocyte production, whereas in another study no phenotype was observed[94,95]. However, in both studies, a dramatic and premature transformation of sporozoites into exoerythrocytic forms in the mosquito was observed. In *Plasmodium yoelii* (another rodent parasite), *pypuf2* disruption also leads to premature sporozoite transformation but does not affect gametocyte development[97]. Considering the profound differences in gametocyte development in *P. falciparum* and rodent parasite species, it would not be surprising if the function of Puf2 has diverged between these species.

Gametocytogenesis and links to virulence

It has been suggested that expression of *pfap2-g* may be mutually exclusive with that of the *var* genes[24,50], which encode variants of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is involved in cytoadhesion and antigenic variation, and the strict expression of only one *var* gene at a time (known as mutually exclusive expression) is regulated in part through epigenetic mechanisms (BOX 3). Several unusual features of *var* genes that have been implicated in their regulation are shared with *pfap2-g*. First, as with the *var* genes, the *pfap2-g* locus has a perinuclear localization in most cells and is enriched in H3K9me3 and PfHP1 in bulk cell population measurements[79,98]. Next, *pfap2-g* and the *var* genes are regulated by PfHP1 and PfHda2, such that depletion of these proteins leads to increased *var* gene expression, increased *pfap2-g* expression and increased gametocyte formation[78,80]. Intriguingly, the *pfap2-g* locus also has two insulator-like pairing elements, consisting of a TG-rich DNA sequence, that are otherwise largely restricted to *var* genes and are thought to be required for silencing[50,99]. It has been demonstrated that these pairing elements are bound by an as-yet-unidentified nuclear protein, which may be important for silencing[99].

If *pfap2-g* and the *var* genes are indeed regulated in a mutually exclusive manner, one would expect cells expressing PfAP2-G (that is, committed cells and gametocytes) not to express *var* genes. Although there are conflicting reports[100,101], recent work using purified gametocytes has shown that the *var* genes are strongly downregulated in stage II gametocytes compared with ring-stage parasites[102]. This is puzzling, as gametocytes sequester in the bone marrow, although it is unclear whether this sequestration is mediated by PfEMP1. This sequestration could involve surface molecules other that PfEMP1, such as RIFIN proteins, which are encoded by the *rif* multigene family[103,104]. Many RIFINs are exported to the red blood cell surface by asexual parasites and are expressed in gametocytes[103,104]. RIFINs have recently been shown to be involved in asexual parasite adherence to the microvasculature, although a role in gametocyte sequestration remains to be explored[105]. Nonetheless, evidence for this mechanism of mutually exclusive expression of *pfap2-g* with *var* genes is currently lacking and will require further investigation. If correct, this would provide an elegant example of epigenetically mediated switching as a control mechanism for a major cellular differentiation process.

Summary and future directions

Recent discoveries have begun to reveal the molecular mechanisms driving commitment to gametocytogenesis in *Plasmodium* spp. (FIG. 3). Expression of PfAP2-G is crucial for gametocytogenesis in *P. falciparum*, probably because it regulates early gametocyte genes and thus initiates the transcriptional programme for sexual development. Although the mechanisms that lead to activation of *pfap2-g* are yet to be fully elucidated, epigenetic regulation is evidently crucial, as depletion of either PfHP1 or PfHda2 leads to upregulation of *pfap2-g* and a dramatic increase in gametocyte production. In a population of *P. falciparum*-infected red blood cells, only a small number of parasites commit to form gametocytes per 48-hour cycle of development; potentially, only those cells expressing sufficient *pfap2-g* become committed following derepression of the *pfap2-g* locus. Elevated levels of PfAP2-G could also be due to post-transcriptional regulation of the *pfap2-g* transcript, for example owing to decreased expression or activity of PfPuf2, thereby adding another potential level of complexity to the regulation of gametocytogenesis.

Nonetheless, many open questions remain. Although several studies have examined transcription in gametocytes, the transcriptional changes that occur during commitment have proved difficult to study, in part owing to the paucity of known early gametocyte markers that could be used to sort committed from uncommitted cells using flow cytometry. Microarray analysis of parasites in which PfHP1 or PfHda2 have been depleted provide some insight into the transcriptional changes occurring during commitment and early gametocyte development[78,80]. However, only one gene (PF3D7_0832300/MAL7P1.224) apart from *pfap2-g* was found to be upregulated during commitment in PfHP1-depleted cells[78]. The identification of PfAP2-G as a pivotal factor that is required for gametocytogenesis and is expressed in committed cells makes purifying committed cells more accessible. Although several cell lines expressing GFP-tagged gametocyte-specific proteins or GFP under the control of gametocyte-specific promoters have been described[46,53,106–109], these proteins are probably expressed downstream of PfAP2-G. Cell lines expressing fluorescently tagged PfAP2-G would be a useful tool for sorting cells that express PfAP2-G from those that do not,

RNA fluorescence *in situ* hybridization
A technique used to visualize the location of a specific mRNA in the cell by using nucleic acid probes complementary to the target that are coupled to a fluorescent molecule.

and thus for sorting committed cells from those that will continue asexual development.

Many questions regarding the timing and requirements for commitment remain. As only a small proportion of the population commits to sexual development, bulk-analysis methods are not ideal for examining this process. Thus, single-cell studies will be essential for dissecting the cellular requirements for commitment and for gaining a better understanding of the level of transcriptional variation between cells. Several genes expressed in committed schizonts and early gametocytes have now been identified, and determining the hierarchy of gene expression in these cells will be a priority. RNA fluorescence *in situ* hybridization, which is already well established in *P. falciparum* research, will be an invaluable tool in this regard. Furthermore, time-lapse single-cell microscopy will allow the fates of individual cells to be monitored, enabling the dynamics of expression of PfAP2-G and other fluorescently tagged gametocyte proteins to be investigated. One major question that remains unanswered is: what leads certain cells to commit to sexual development? One possible model is that PfAP2-G is expressed in a stochastic manner and that a threshold of PfAP2-G is required for commitment to occur. Given that AP2-G positively regulates itself, stochastic expression would explain why only a small proportion of cells commit to gametocyte development. Again, single-cell studies will be required to test this hypothesis.

An additional remaining puzzle is how environmental cues known to increase rates of commitment are translated into the molecular signals that initiate gametocyte development. PfAP2-G expression now provides a possible readout for commitment, although it remains to be shown whether the environmental cues that promote gametocytogenesis are mediated by PfAP2-G. One possibility is that these environmental signals somehow lead to epigenetic changes at the *pfap2-g* locus, potentially mediated by PfHP1 and PfHda2, which may determine the probability of activation. However, major questions remain: how do these cues originate? Under what conditions does this occur *in vivo*? What are the downstream signalling cascades that report on cellular alterations in response to these cues? Are these key triggers delivered by extracellular vesicles, which have been reported to enhance gametocyte induction[33,34]?

It is exciting to imagine that, through a better understanding of the mechanisms underlying commitment, we may be able to produce parasites that commit to gametocytogenesis at significantly higher rates. Without a doubt, the study of gametocytes and gametocytogenesis has been hampered by the difficulty in isolating gametocytes in large-enough numbers to study them in detail. Controlled production of gametocytes would be immensely valuable to delve much deeper into gametocyte biology. PfAP2-G has already been shown to be required for gametocytogenesis, and derepression of PfAP2-G by PfHP1 and PfHda2 depletion leads to a dramatic increase in the number of gametocytes produced, which suggests that constitutive expression of PfAP2-G may be sufficient to drive gametocytogenesis[50,78,80]. Manipulating expression of PfAP2-G is thus a promising mechanism for increasing gametocytogenesis. As well as revolutionizing the field of gametocyte biology, this could also facilitate the development of transmission-blocking vaccines and gametocytocidal drugs by making this stage more amenable to study and enabling the production of large numbers of purified gametocytes that candidate therapeutics could be tested against. Few widely used antimalarial drugs are highly active against gametocytes[110], which is a major problem, as drugs that kill gametocytes are required to prevent transmission and to ultimately eliminate the disease. Several studies have specifically examined the effect of various compounds on gametocyte development, but these have been hampered by factors such as sex-specific differences in response to drugs, the use of different parasite stages and different readouts for assessing efficacy[65,110–116].

Having a greater understanding of the molecular pathways occurring in committed cells and during early gametocytogenesis may also be useful in the field. Gametocytes are often present at submicroscopic levels, which are nonetheless sufficient for transmission to the mosquito[117]; thus, reverse transcription PCR-based methods must be used to reliably identify individuals that carry gametocytes[118]. Although mature gametocytes can be detected using the 25 kDa ookinete surface antigen gene *pfs25* (PF3D7_1031000/PF10_0303) as a biomarker[119], markers of early gametocytes or even committed cells would also be a useful tool. Some early gametocyte genes have already been proposed as potential candidates[52], and one of these (PF3D7_1477700/PF14_0748) has been used in the field to assess the prevalence of early gametocytes[39,41].

In closing, recent molecular discoveries regarding how commitment to sexual differentiation is regulated have considerably deepened our understanding of *Plasmodium* spp. gametocyte biology. They not only shed light on the longstanding question of how the decision to form gametocytes occurs but also will enable the investigation of the events that occur in committed cells. Importantly, these findings suggest new tools that will allow us to address remaining open questions surrounding sexual differentiation and help move research towards the long-desired goal of subverting the malaria parasite.

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Competing interests statement
The authors declare no competing interests.

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