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Regulation of Sexual Commitment and Gametocytogenesis in Malaria Parasites

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Abstract

Sexual differentiation of malaria parasites from the asexual blood stage into gametocytes is an essential part of the life cycle, as gametocytes are the form that is taken up by the mosquito host. Because of the essentiality of this process for transmission to the mosquito, gametocytogenesis is an extremely attractive target for therapeutic interventions. The subject of this review is the considerable progress that has been made in recent years in elucidating the molecular mechanisms governing this important differentiation process. In particular, a number of critical transcription factors and epigenetic regulators have emerged as crucial elements in the regulation of commitment. The identification of these factors has allowed us to understand better than ever before the events occurring prior to and during commitment to sexual development and offers potential for new therapeutic interventions.

Keywords

malaria; *Plasmodium*; gametocyte; AP2-G; sexual development; gametocytogenesis; malaria pathogenesis

BACKGROUND

Malaria is a major global health concern, resulting in over two million infections and 445,000 deaths in 2016 (83). The disease is caused by unicellular protists belonging to the genus *Plasmodium*, and five species are known to infect humans (*P. falciparum, P. vivax, P.*

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ovale, P. malariae, and *P. knowlesi*). Of these, *P. falciparum* and *P. vivax* cause the majority of human infections, though *P. falciparum* is the most virulent. *Plasmodium* parasites have a complex life cycle that involves development in both the human host and the *Anopheles* mosquito.

The observation in 1880 by Charles Louis Alphonse Laveran (41) of an activated male, sexual stage parasite in an unstained blood sample from a patient that had been febrile 15 days before was the first strong evidence of the cause of malaria. This critical observation was extended over the next 17 years, culminating in the demonstration by Ronald Ross (65) that these sexual forms were required for transmission via a mosquito through the population. Since that time, spurred on by the development of cell stains to track parasites, a robust culture system, and new omics approaches, we have learned much about the complex life cycle of malaria parasites and their pathogenicity. However, a detailed understanding of the mechanisms underlying the regulation of the balance between asexual replication that expands the intrahost parasite population and sexual differentiation that allows parasite transmission through the population has lagged. Recent studies have identified a number of genes involved in the regulation of gametocytogenesis and also revealed the important contribution of epigenetic factors and metabolic influences, thus greatly advancing our understanding of this essential process.

THE MALARIA PARASITE LIFE CYCLE

The *P. falciparum* life cycle is shown in Figure 1. Both asexual and sexual differentiation and development take place during the intraerythrocytic portion of the life cycle. This begins with the release of merozoites from a liver cell that was infected with a sporozoite 2–7 days before, when the host was bitten by an infected mosquito. These hepatic merozoites are subsequently released into the blood, where they invade erythrocytes. Once in the erythrocyte, *Plasmodium* parasites replicate asexually, producing merozoites that will either reinitiate asexual development or commit to sexual differentiation during the next erythrocytic cycle. This decision to begin sexual development is thought to occur prior to schizogony (9). In *P. falciparum*, asexual replication takes 48 h and produces 16–32 new merozoites, whereas sexual differentiation results in the development of a single male or female gametocyte over the course of 10–12 days. This prolonged time course of sexual development explains why Laveran did not see mature, activated gametocytes until 15 days after the patient was first febrile and presumably had high levels of asexual parasites. In vitro culture allows the direct observation of gametocytes maturing through five morphologically distinct stages (I–V) (33); however, in vivo, both mature *P. falciparum* asexual parasites and immature stage I–IV gametocytes are sequestered and are therefore not observed in peripheral blood samples (35). Importantly, in mouse models of malaria (such as *P. berghei* and *P. yoelii*) there are several key differences in gametocytogenesis (described in Table 1), including a much shorter 30-h maturation time. During maturation to stage Vgametocytes, the parasites become more deformable and return into circulation (76), where they can be taken up in a blood meal by a mosquito and can be detected in a blood smear. For transmission, both a male and a female must be taken up by a mosquito, activate, fertilize, and produce an oocyst, where tens of thousands of sporozoites are produced. Once released

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from the oocyst, about 12 days later, the sporozoites migrate to the salivary gland, where they can be transmitted to another human during a blood meal.

GAMETOCYTOGENESIS

Based on early work in *P. falciparum*, it is widely believed that commitment likely occurs shortly before schizogony, with every merozoite in a schizont forming either a gametocyte or an asexual parasite but never a combination (9). Similarly, every merozoite in a schizont will form either a male or a female gametocyte, but never a mix of both (67, 72). This indicates that commitment and sex determination both occur before the schizont stage.

Only a subpopulation of schizonts commit to gametocyte production during an intraerythrocytic cycle. Furthermore, the level of gametocytes produced varies between and within clonal parasite lines, suggesting that both genetic and epigenetic factors are involved. In *P. falciparum*, typically fewer than 10% of the parasites produce gametocytes in each 48-h cycle. This is in stark contrast to *Haemoproteus* spp., where merozoite invasion of red blood cells is characterized by full conversion to gametocytes (73). Therefore, dissecting how *Plasmodium* parasites modulate commitment rate is critical to understanding parasite transmission.

In the last five years there have been major advances through in vitro studies that have provided insight into *Plasmodium* gametocytogenesis and the regulation of commitment to the sexual differentiation fate. Though various aspects of gametocytogenesis have been the subject of several comprehensive reviews (1–5, 19, 21, 32, 36, 42, 54, 55, 69, 70, 74), here we focus on these emerging insights and describe how they are shaping our understanding of malaria parasite transmission, with a particular focus on *P. falciparum*.

KEY GENETIC REGULATORS OF COMMITMENT

The key drivers of gametocytogenesis were long unknown, but forward genetics has recently provided considerable insight into how this critical process is regulated. It is common for laboratory strains to lose the ability to make gametocytes; when parasites are continuously cultured there is no selection for the ability to form sexual stages. One of these gametocyte-deficient lines was shown to have a deletion of a region of chromosome 9, and subsequently the gene responsible was identified as*gdv1* (gametocyte development 1) (PF3D7_0935400/PFI1710w) (23). Overexpression of GDV1 results in an increase in gametocyte production, and it is present in the nucleus; this implicated it as a possible transcriptional regulator of commitment. Subsequent work has further characterized GDV1 as a likely epigenetic regulator (24) and is discussed further below.

Later, two groups independently sequenced strains of human (*P. falciparum*) and rodent (*P. berghei*) malaria parasites that had lost the ability to form gametocytes (37, 71). These strains all had mutations in a gene encoding the protein PF3D7_1222600/PFL1085w (now known as AP2-G, for ApiAP2 involved in gametocytogenesis). AP2-G is a member of the ApiAP2 family of DNA-binding proteins. This family is related to the plant AP2/ERF transcription factor family and is the major group of transcription factors in *Plasmodium* (56). Reverse genetics has confirmed that AP2-G is a key regulator of gametocytogenesis; in

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the absence of AP2-G, parasites do not produce gametocytes (37, 71). Conversely, overexpression of *ap2-g* in *P. berghei* by using a strong constitutive promoter leads to a dramatic and rapid increase in gametocyte production (39). This ability to change cell fate through overexpression of a transcription factor is a hallmark of a master regulator. Crucially, parasites in which *ap2-g* has been disrupted have a growth advantage over wild-type parasites (15, 37, 52). This is consistent with AP2-G regulating the commitment phase, since parasites that are unable to commit will not divert from asexual multiplication to enter gametocytogenesis and thus will outcompete gametocyte-producing parasites. AP2-G has a single AP2 DNA-binding domain that in vitro binds the GNGTAC motif that is found in the promoters of many gametocyte genes (37). However, it is unknown whether AP2-G binds these sites in vivo and which genes it directly regulates. These data will be necessary to confirm exactly how AP2-G transcriptionally regulates gametocytogenesis.

ENVIRONMENTAL AND METABOLIC EFFECTS ON COMMITMENT

Many factors have been identified that seemingly affect commitment rates, including spent media (82), the presence of reticulocytes (77), and antimalarial drugs (11). Cholera toxin has also been reported to increase commitment rates (21), though its mechanism is not understood (58). Methods to induce commitment in laboratory strains generally rely upon stressing the culture by using spent media (7, 25, 64). Despite early work hinting at possible signaling pathways involved in triggering commitment (21, 38), the molecular mechanisms that connect these environmental signals to molecular processes in the cell have remained unclear (10).

Recently it was reported that lysophosphatidylcholine (LysoPC) can modulate gametocyte formation (8). LysoPC is a common component of human serum; depletion of LysoPC results in enhanced gametocyte production in vitro. As such, it is the first defined environmental signal that acts as an inhibitor of gametocyte production. LysoPC is thought to regulate commitment not by acting as a direct trigger of a signaling cascade but rather by causing metabolic changes in the cell that might influence epigenetic regulation. The action of LysoPC could explain several earlier reports describing enhanced gametocyte production with the use of conditioned media (8), which might have reduced levels of LysoPC. It is also possible that parasites sequestered in locations with lower LysoPC concentrations (such as the bone marrow) are more likely to commit to sexual differentiation.

Though environmental factors evidently influence commitment rate, it is likely that sexual differentiation is an intrinsic feature of intraerythrocytic development and that there is a basal level of gametocytogenesis that is regulated by stochastic expression of *ap2-g*. Based on this hypothesis, the differences observed between distinct clonal parasite lines are due to subtle genetic or epigenetic differences that regulate *ap2-g* expression (37). In this scenario gametocytes would be constantly produced at a low level during each intraerythrocytic cycle. This model is consistent with current in vitro and in vivo evidence and suggests that observed variation in gametocyte production is due to intrinsic variation in the stochastic expression of *ap2-g*. More work is needed to define the genetic and epigenetic components contributing to *ap2-g* expression and to determine what influence external factors have on regulating this basal, stochastically varying rate. It is likely that there is a complex interplay

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of factors, with important roles for both environmental signals and intrinsic capacity to commit.

TRANSCRIPTIONAL REGULATION

Precommitment

Though AP2-G itself plays a crucial role in regulating commitment, it remains unknown which transcription factors are responsible for directing transcription of *ap2-g* itself. One potential candidate is PF3D7_1317200/PF13_0097, which is another ApiAP2 protein. PF3D7_1317200 was initially implicated in the regulation of gametocytogenesis through a piggyBac mutagenesis screen, though its importance has not yet been validated using either complementation or independent disruption of the gene (34). An insertion in the coding region of PF3D7_1317200 resulted in loss of gametocytogenesis, though several early gametocyte markers were still transcribed. Intriguingly, PF3D7_1317200 transcript levels are not reduced when *ap2-g* is disrupted (37). This suggests that PF3D7_1317200 might be acting upstream of AP2-G and could be its direct activator. Consistent with this model, disruption of the *P.yoelii* ortholog of PF3D7_1317200 (named PyAP2-G3) leads to significantly reduced numbers of gametocytes (88). Expression of *ap2-g* is reduced in the knockout, but as in *P. falciparum* disruption of *ap2-g* does not affect levels of the *ap2-g3* transcript. PyAP2-G3 is present in both the nucleus and the cytosol, suggesting that it might be able to shuttle between the two compartments in response to environmental signals. This would provide a potential link between environmental factors known to affect commitment and transcription of *ap2-g*. Interestingly, the *P. berghei* ortholog of PF3D7_1317200 has not been disrupted despite multiple attempts (52), though it is unclear whether this reflects a genuine biological difference between these two rodent malaria parasite species or is simply due to technical differences between the two studies (CRISPR-Cas9 versus PlasmoGEM-based approaches). Further investigation of the function of PF3D7_1317200 and its orthologs in other species will be necessary to clarify its role.

Postcommitment

As the only family of transcription factors to have been identified in *Plasmodium*, members of the ApiAP2 family are the main candidates for transcription factors acting downstream of AP2-G. AP2-G2 is important in later gametocyte development in the rodent malaria parasites *P. berghei* and *P. yoelii*, and its disruption leads to nearly complete loss of mature gametocytes (71, 88). Sinha et al. (71) showed that this results in the total loss of males but that some females survive, though later work by another group suggests that parasites expressing male gametocyte markers and those expressing female gametocyte markers are both present at reduced levels (85). AP2-G2 is expressed in male and female gametocytes, as well as asexual stages. Unlike AP2-G, its main role appears to be in repressing transcription. Although disruption of AP2-G2 leads to reduced levels of gametocyte-specific transcripts (71, 85), AP2-G2 does not regulate these genes directly. ChIP-seq experiments have shown that it is associated with the promoters not of gametocyte genes, but rather of genes expressed primarily in the asexual stages as well as the mosquito and liver stages (85). Reporter assays have confirmed that AP2-G2 binding is associated with repression. The defect in producing gametocytes seen in parasites in which AP2-G2 has been disrupted is

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thought to result from the inappropriate transcription of stage-specific genes caused by lack of repression, resulting in aberrant development.

Several other ApiAP2 proteins have been implicated in the regulation of gametocytogenesis; these (and other potential regulators) are summarized in Table 2. Poran et al. (60) showed using scRNA-seq that the ApiAP2 proteins PF3D7_1222400/PFL1075w and PF3D7_1139300/PF11_0404 are both upregulated in AP2-G-expressing cells. Interestingly, PF3D7_1222400 has no ortholog in rodent malaria species, and lab strains are particularly prone to acquiring mutations in PF3D7_1222400, suggesting it is dispensable for asexual stages (16). This is consistent with a possible role in gametocytogenesis.

Two studies measuring nascent transcription in gametocytes have also identified a number of potential transcriptional regulators. Early in gametocytogenesis, the transcripts encoding the ApiAP2 proteins SIP2 (PF3D7_0604100/PFF0200c) and AP2-O (PF3D7_1143100/PF11_0442) are stabilized (57). Later in gametocytogenesis, both AP2-O and PF3D7_1429200/PF14_0271 are transcribed (45). SIP2 is involved in heterochromatin formation, and AP2-O is a regulator of ookinete genes in *P. berghei* (26, 87). Little is known about PF3D7_1429200 beyond the fact that it can be disrupted and is thus dispensable for blood stages (46). Its single AP2 domain has no known DNA motif, and disruption of the *P. berghei* ortholog leads to a severe defect in ookinete formation (52). Further studies will be required to determine the functional role of these ApiAP2 proteins in sexual commitment as well as developmental cell fate decisions and the regulation of differentiation during gametocytogenesis.

EPIGENETIC REGULATION

Precommitment

Epigenetic regulation has been well studied in asexual stages, where it is known to be important in regulating genes involved in critical processes such as cytoadhesion, immune evasion, and solute transport (20), and several histone modifications have also been mapped in oocysts and sporozoites (31). In addition, a wide range of histone posttranslational modifications have been identified in *P. falciparum* and are likely to play important distinct regulatory roles (17, 49, 78).

One well-characterized histone mark in *P. falciparum* is H3K9me3, which has been shown to play a key role repressing expression of subtelomeric gene families as well as some of the genes associated with gametocytogenesis (44). The initial *P. falciparum* ChIP-chip analysis showed that H3K9me3 is restricted primarily to regions containing certain multigene families (especially in subtelomeric regions) that are generally silenced (44). H3K9me3 recruits heterochromatin protein 1 (HP1), and thus the two have very similar distributions genomewide (27, 28). Interestingly, the *ap2-g* locus is associated with H3K9me3 and HP1, indicating that in the majority of cells *ap2-g* is epigenetically silenced. To determine the functional role of HP1 an inducible degradation system was used to deplete HP1 at specific times during intraerythrocytic development (6). Depletion of HP1 expression from ~30–40 h after erythrocyte invasion resulted in 50% of the next generation of parasites differentiating into gametocytes instead of replicating asexually, suggesting that HP1 plays a key role

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repressing the production of gametocyte-committed merozoites during schizogony. Transcriptional analysis comparing development in the presence and absence of HP1 demonstrated an increase in transcripts associated with early gametocytes, with one of the earliest changes being an increase in *ap2-g* RNA levels. Such an increase in *ap2-g* transcription would be sufficient to induce gametocyte-committed merozoite production, although it is possible that there were additional *ap2-g*-independent changes in gene transcription mediated by HP1. No HPl-dependent change was observed in expression levels of *gdv1*, the other gene shown to regulate gametocyte production, consistent with it not being enriched for H3K9me3 and suggesting it is independent of *ap2-g* expression.

Recent work suggests that GDV1 is involved in the removal of HP1 from *ap2-g*, resulting in derepression of *ap2-g* and allowing cells to commit (24). GDV1 directly interacts with HP1, and the two factors are enriched at most of the same loci genomewide. Overexpression of GDV1 leads to decreased HP1 occupancy at specific loci, including *ap2-g*, resulting in derepression of *ap2-g* (and a small number of other genes) and greatly increased commitment rates (~50%). Interestingly, levels of GDV1 appear to be controlled at least in part via the action of a *gdv1* antisense transcript, as deletion of the region encoding this transcript results in upregulation of *ap2-g*.

Like HP1, the histone deacetylase Hda2 has also been shown to regulate commitment by contributing to epigenetic silencing of the *ap2-g* locus (18). Conditional depletion of Hda2 leads to a twofold increase in gametocyte production. Hda2 is most likely required to decrease histone acetylation during DNA replication to facilitate H3K9me3 and gene repression in the subsequent generation. Additional work is still needed to identify the full complex of regulatory factors involved, including the specific lysine methyltransferases and demethylases that establish the normal balance of asexual and sexual differentiation. Identifying the genes involved in modulating *ap2-g* expression would provide specific targets that could be influenced by external signals or possibly novel drugs to modulate gametocyte production.

These findings strongly suggest that H3K9me3, HP1, and Hda2 are required to repress sexual differentiation and allow asexual replication to amplify the number of erythrocytic parasites. However, they do not explain how *ap2-g* expression is regulated by external signals. One possible link between epigenetic regulation and external cues is through LysoPC in the host serum. It has been demonstrated in vitro that when LysoPC is present, it is metabolized to choline and finally phosphatidylcholine (PC) via the Kennedy pathway (8). However, in the absence of LysoPC, metabolic enzymes involved in S-adenosylmethionine (SAM) metabolism are transcriptionally upregulated. This transcriptional upregulation likely occurs because the cell needs to continue to produce PC but must now switch to an alternative route such as converting serine or ethanolamine into choline. Key to this conversion is the enzyme phosphoethanolamine methyltransferase, which uses a SAM-dependent three-step methylation reaction to convert phosphoethanolamine into phosphocholine. However, SAM is also critical to a number of histone methyltransferase enzymes in the nucleus. Therefore, a shift in the pool of SAM to PC production could result in less SAM being available in the nucleus for histone methyltransferases and thus a decrease in histone methylation. This could lead to derepression of the *ap2-g* locus (and

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other heterochromatic loci) and thus increased levels of sexual commitment. However, why this derepression only occurs in a subpopulation of cells remains an important open question.

Postcommitment

Although relatively little work has examined epigenetic regulation during gametocyte development, it is likely that it is also important during this crucial transition. Nucleosome positioning in stage IV–V gametocytes has been determined using MAINE-seq, and this revealed that gametocytes have a relatively open chromatin structure (14). As in other stages, the majority of nucleosomes are located in gene bodies whereas intergenic regions are generally relatively depleted of nucleosomes. Chromosome conformation capture has also been used to examine genome organization in both early and late gametocytes, revealing significant changes in chromatin organization during gametocytogenesis (12).

Recently, quantitative proteomics was used to identify and quantify histone modifications present throughout all five stages of gametocytogenesis as well as three stages of the intraerythrocytic cycle (17). Fifteen modifications previously unidentified in *P. falciparum* were found, and several of these appear to be specific to or highly enriched in gametocytes. This includes H3K27me3, which is a modification generally associated with silencing that has long been thought to be absent in *Plasmodium*. This modification—as well as several other putative repressive marks—was highly enriched in stage II gametocytes, suggesting that repression of gene expression might be a hallmark of early gametocytes. It is possible that as the gametocyte transcriptional program is being initiated, asexual-stage genes are also being turned off. In contrast, euchromatic marks are more abundant in late-stage gametocytes. This observation is consistent with the nucleosome positioning data, which suggest that the late gametocyte chromatin structure is fairly open and euchromatic and is overall suggestive of abundant transcription (or perhaps preparation for replication). Mapping of the identified modifications across the genome in various stages of gametocyte development will be essential to understanding their role during gametocytogenesis.

Early (stage II/III) and late (stage IV/V) gametocytes have significant differences in HP1 occupancy compared with asexual parasites (28). A small number of genes (including *ap2-g*) that are normally heterochromatic in asexual parasites are no longer so in gametocytes. Conversely, many genes (such as some of those encoding proteins that are involved in remodeling of the host cell) become enriched in HP1 during gametocytogenesis.

A number of potential epigenetic regulators that are upregulated in committed cells have been identified (6, 8, 60). Hda1, a putative histone deacetylase, has been shown in several studies to be expressed following induction of AP2-G (6, 8, 60). Though Hda1 has not yet been characterized, histone deacetylases often act as repressors. A newly identified histone demethylase (LSD2) and the putative chromatin remodelers ISWI and SNF2L are similarly upregulated in AP2-G-expressing cells (60). Although none of these has been extensively characterized in *Plasmodium* parasites, ISWI localizes to the central region of the nucleus that predominantly contains euchromatin (79).

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POSTTRANSCRIPTIONAL REGULATION

Precommitment

Little is known about posttranscriptional regulation prior to commitment. However, recently an investigation of nascent transcription in committed cells revealed that early gametocyte genes are actively transcribed even in the F12 line, which does not express functional AP2-G (57). In contrast to the parental 3D7 line, which can make gametocytes, these transcripts are not stable in F12. This may account for why previous studies have shown a reduction in levels of these transcripts in F12 (30, 68); though these genes are being transcribed at levels similar to the wild-type line, the transcripts are rapidly degraded and are thus not readily detectable using steady-state approaches. These data suggest not only that transcriptional regulators independent or upstream of AP2-G are involved in the transcription of early gametocyte genes (potentially in a precommitment step) but also that posttranscriptional regulation plays a critical role in the regulation of commitment. Evidently transcription of early gametocyte genes is not sufficient for cells to commence gametocytogenesis, as these transcripts also need to be stabilized. *Plasmodium* parasites encode a wide array of RNA-binding proteins, with estimates ranging as high as 15–20% of protein-coding genes containing predicted RNA recognition motifs (13, 62). Identification of RNA-binding proteins that are involved in stabilizing these transcripts and allowing the cell to commit to sexual differentiation will be crucial to future studies.

Postcommitment

Posttranscriptional regulation is also important in gametocyte development. Many well-established gametocyte genes are not necessarily transcribed at a higher level in committed cells; rather, their transcripts are more stable, and thus they accumulate (57). Stabilization of gametocyte transcripts by RNA-binding proteins is thus likely an important mechanism for regulating the early stages of gametocytogenesis. Posttranscriptional regulation is evidently also important later in gametocyte development, as a recent study comparing the proteomes and transcriptomes of stage V gametocytes found many mRNA transcripts that are present without their corresponding protein products, suggesting that these transcripts are being translationally repressed and stored for translation later in the life cycle (40).

Two members of the Puf family of RNA-binding proteins have been characterized in *Plasmodium*, and both are involved in regulating gametocytogenesis. Puf proteins are found in many diverse organisms and are generally translational repressors; that is, they bind transcripts and prevent their translation. In *P. falciparum*, disruption of PfPuf2 leads to an increase in gametocyte production, so PfPuf2 acts as a repressor of sexual development (51). The protein is able to interact with the 5′ and 3′ untranslated regions of sexual-stage transcripts, and this interaction leads to translational repression (50). When Puf2 is no longer present, this repression no longer occurs and more gametocytes can form. Recently, PfPuf1 has also been implicated in gametocytogenesis (66), as knockout of *pfpuf1* leads to decreased gametocytogenesis. These parasites develop normally until stage III and then begin to disappear (presumably because they die). Though the mechanism by which Puf1 regulates gametocytogenesis has not yet been elucidated, given that it is a Puf family protein, it likely also acts as a translational repressor.

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Another RNA-binding protein—ALBA4—has also been shown to play a role in regulating male gametocyte development in *P. yoelii* (53). ALBA4 is a member of the ALBA family (acetylation lowers binding efficiency) that binds nucleic acids. Disruption of *alba4* leads to increased centers of movement but no increase in the number of males, indicating increased exflagellation. The mechanism by which ALBA4 regulates exflagellation and the transcripts it interacts with are unknown, but its disruption is known to lead to changes in the levels of hundreds of transcripts, and in gametocytes it interacts with many other RNA-binding proteins, including other ALBAs.

TRANSCRIPTOMICS IN GAMETOCYTES AND COMMITTED CELLS

Historically, examining transcription throughout gametocytogenesis (and in particular during the early stages) has been challenging due to the difficulty of isolating sufficient numbers of cells for analysis. One popular approach has been to compare transcript levels in a gametocyte-deficient line (such as F12) to those of its parent and infer that any genes expressed at a higher level in the parent are gametocyte genes (22, 23, 30, 68).

Several studies have also examined the transcriptomes of gametocytes at various developmental stages. For example, Young et al. (84) used microarrays to identify a set of 246 genes that are upregulated during gametocyte development. RNA-seq has also been used to assess the transcriptomes of stage II and stage V gametocytes (43), as well as separated male and female stage IV/V gametocytes (40). The latter work revealed that the majority of gametocyte genes are expressed in a sex-specific manner. From these studies and others, several genes that are considered reliable gametocyte markers have emerged. These include *pfs16, pfg27, pfg14.744, pfg14.748, etramp10.3/peg4*, and, most recently, *gexp05*. AP2-G is able to bind the promoters of two of these (*pfg27* and *etramp10.3*) and drive transcription in vitro (37), but others may be regulated in an AP2-G-independent manner. One example is GEXP05, which is expressed even in the F12 line (75). This suggests that while AP2-G may regulate a subset of gametocyte genes early in commitment, other genes may be regulated by additional factors.

One group has recently purified cells expressing a reporter whose expression is driven by a gametocyte-specific promoter (*etramp10.3*) and examined their transcriptomes using microarrays (59). Importantly, *etramp10.3* is expressed in committed schizonts and until stage II in gametocytes, allowing for the first-ever examination of the committed schizont transcriptome. This approach found 308 transcripts that are differentially expressed in committed cells and early gametocytes, including *ap2-g*. By looking for genes with similar transcription patterns to *ap2-g*, further putative early markers of gametocytogenesis were also identified.

Others have measured transcript levels following gametocyte induction by perturbing expression of regulators that function upstream of *ap2-g*. As both HP1 and Hda2 are involved in repressing gametocytogenesis, parasite lines engineered to allow conditional degradation of these proteins can be used to produce large numbers of gametocytes (6, 18). Conversely, overexpression of GDV1 leads to significantly increased gametocyte production (24). Because these systems are conditional, this allows for control over the timing of

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commitment and thus dissection of the timing of gene expression during early gametocyte development (including *ap2-g*) as well as the identification of many novel early gametocyte markers. Although most early gametocyte genes appear to be regulated by AP2-G, there are also a small number of genes that are upregulated following overexpression of GDV1 even in the F12 parasite line. This suggests that GDV1 regulates commitment not only via direct activation of *ap2-g* but also via its regulation of other gametocyte genes. Interestingly, fairly few genes have been identified that are upregulated in the committed schizont phase compared to committed rings or stage I gametocytes, perhaps reflecting that committed schizonts still need to perform many of the same functions as asexual schizonts (6, 57). Following reinvasion and the development of sexual rings, much more abundant transcriptional changes seem to occur.

Brancucci et al. (8) recently used LysoPC to inhibit commitment and compared transcript levels to those of a parallel culture grown without LysoPC. This has revealed many transcripts that may be upregulated in early gametocytes, including potential transcriptional regulators that could be involved in regulating commitment and gametocytogenesis. Because LysoPC acts upstream of AP2-G, this approach has also uncovered some of the events occurring upstream of commitment. This includes the expression of potential activators of the *ap2-g* locus, such as GDV1. Interestingly, many kinases were upregulated in cells not treated with LysoPC compared to those that were, suggesting that phosphorylation may be important in regulating commitment and early gametocyte development. This work provides clues as to how environmental signals may ultimately be translated in the cells to lead to expression of *ap2-g*. However, because LysoPC depletion also has effects on cells that are unrelated to commitment, it is difficult to disentangle which transcriptional changes are relevant to gametocytogenesis and which are more general responses.

One way to get around this problem is to isolate and study single cells. This approach is particularly well suited to the study of gametocytogenesis, as in a population of cells only a minority will commit. Comparisons between populations are therefore likely to capture only the most dramatic differences as the signal from gametocytes is diluted. To date, two studies have used scRNA-seq to examine transcriptional changes in committed cells and gametocytes (60, 63). By identifying significantly upregulated transcripts in AP2-G-expressing cells, Poran et al. (60) were able to examine the committed schizont transcriptome. They identified many likely transcriptional and epigenetic regulators, as discussed above (and in Table 2). Reid et al. (63) instead focused on stage V gametocytes as well as *P. berghei* gametocytes and reported transcriptional differences between male and female gametocytes, including many *apiap2* genes and members of multigene families. To identify new sex-specific markers, another group has used single-cell qRT-PCR to examine differences in the levels of 91 handpicked transcripts between male and female gametocytes (80). Single-cell studies are in their infancy, but their ability to identify genes expressed early in gametocyte development has provided further insight into the earliest events of commitment and gametocytogenesis and strongly supports the continued use of single-cell approaches as they mature.

To date, only two studies have measured nascent transcription in gametocytes rather than steady-state transcript levels. One approach used biosynthetic labeling of nascent RNA using

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an enzyme under the control of stage-specific promoters (the constitutive *calmodulin* promoter or the gametocyte-specific *pfs16* promoter) to purify transcripts that are more highly transcribed in committed schizonts and early gametocytes and analyze these by microarray (57). By analyzing the unlabeled transcripts as well, they also identified stabilized transcripts. They found 808 genes that are either transcribed or stabilized to a greater extent in committed cells, of which the majority are transcripts known to be present in gametocytes. Surprisingly, this analysis revealed that a limited gametocyte transcriptional program also initiates in F12 parasites, including expression of sex-specific genes. This suggests that sex determination may occur independently of and perhaps even prior to AP2-G expression. Another group used a different method (GRO-seq) to identify nascent transcripts in stage III and stage V gametocytes (45). The set of genes that were more highly transcribed in gametocytes than in asexual stages included many encoding proteins involved in motility, whereas those that were transcribed less included invasion and pathogenesis genes.

FUTURE DIRECTIONS

Though recent work has provided critical insights into the molecular mechanisms underlying commitment and gametocyte development (summarized in Figure 2), many important questions remain. A number of new putative regulators have been identified, and detailed experimentation will be required to determine the role of each in these critical processes. Although various aspects of gametocytogenesis are likely to be regulated at the transcriptional level, a greatly understudied area is the role of RNA-binding proteins. Stabilization and turnover of RNAs may be critical to differentiation, as has already been found for posttranscriptional regulation via translational repression of zygote-specific mRNAs (47). Furthermore, while much of the work to date has focused on mRNAs, additional roles for noncoding mRNAs have not been explored deeply.

Similarly, given the importance of epigenetic regulation to asexual development and commitment, it is very likely to also be important throughout gametocyte maturation. Indeed, we now know that many histone modifications are unique to gametocytes and that these change over time, pointing to a potentially complex epigenetic regulation (17). Future work should focus on understanding the distribution and functions of these modifications, as well as the proteins that deposit them and mediate their effects.

Commitment has long proved very difficult to study due to the small population of committing cells in each cycle and the inability to purify or enrich for these. However, single-cell approaches promise to open up this important stage to study. Some insight has already come from studies using scRNA-seq to identify genes that are differentially expressed in committed cells (60, 63), and further work will continue to fill in some of the details of what is occurring at a molecular level during commitment. This may finally allow us to address some fundamental questions such as how and when sex determination occurs.

We are approaching the point where our understanding of how commitment is regulated will allow us to manipulate *P. falciparum* parasites to commit at dramatically higher rates, as has already been done in *P. berghei* (39). Modulation of epigenetic factors such as HP1 and

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GDV1 has also been shown to increase commitment, though not to 100%, and the identification of more specific regulators of *ap2-g* should allow improvements on this. It will be important to make sure that these manipulations do not cause other effects (such as global derepression of heterochromatic genes), as both HP1 and Hda2 knockdown do. The ability to create large numbers of pure gametocytes will allow us to delve even deeper into gametocyte biology than ever before and make advances in malaria control in the field.

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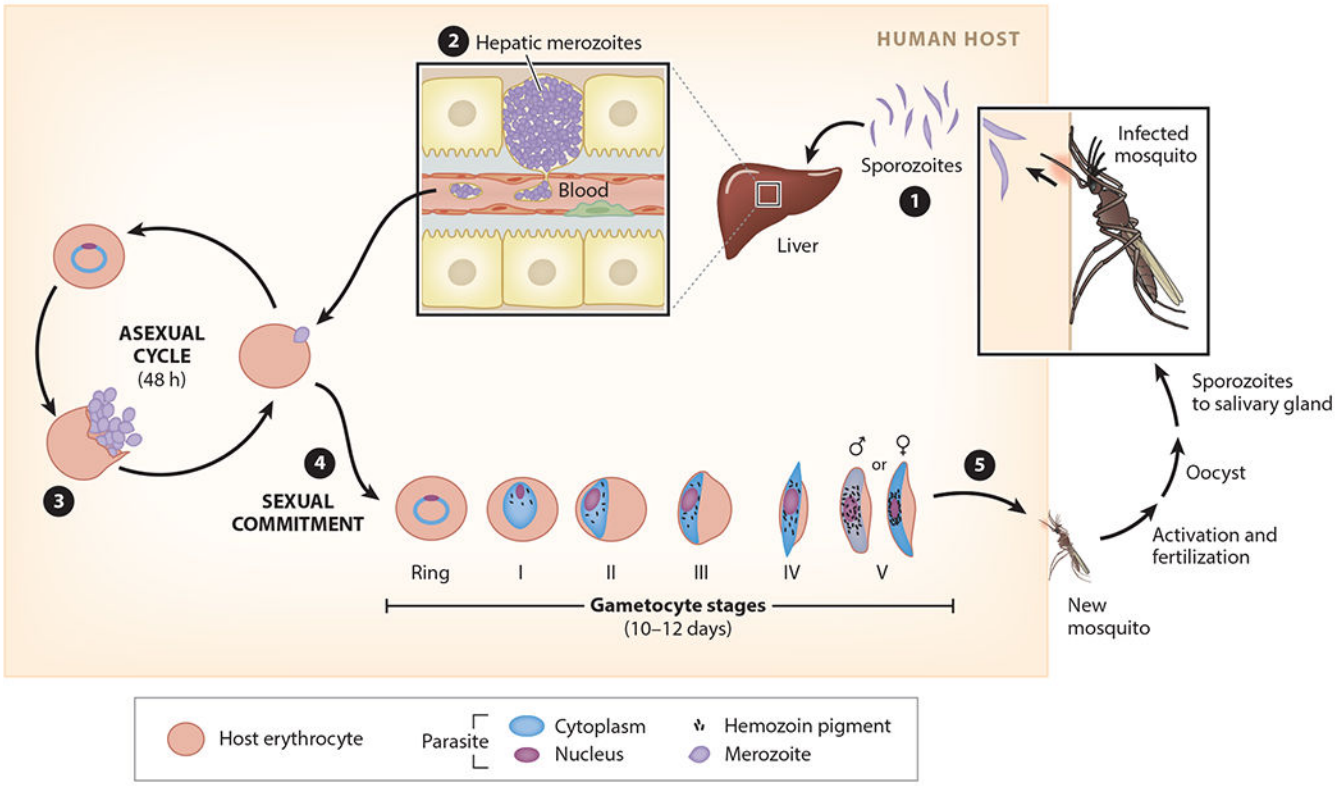

Figure 1.

The life cycle of *Plasmodium falciparum*. (❶) Liver cells are invaded by sporozoites introduced to the human by the bite of an infected mosquito. (❷) Hepatic merozoites develop and are subsequently released into the blood, where they invade erythrocytes. Once in the erythrocyte, *Plasmodium* parasites replicate to produce new merozoites that will either (❸) continue asexual development or (❹) be committed to sexual differentiation into gametocytes during the next erythrocytic cycle. (❺) Late-stage gametocytes can be picked up in a blood meal by a mosquito. For transmission, both a male and a female must be taken up by a mosquito, activate, fertilize, and produce an oocyst, where tens of thousands of sporozoites are produced. Once released from the oocyst about 12 days later, the sporozoites migrate to the salivary gland, where they can be transmitted to another human during a blood meal. Inset ❷ adapted with permission from Reference 61.

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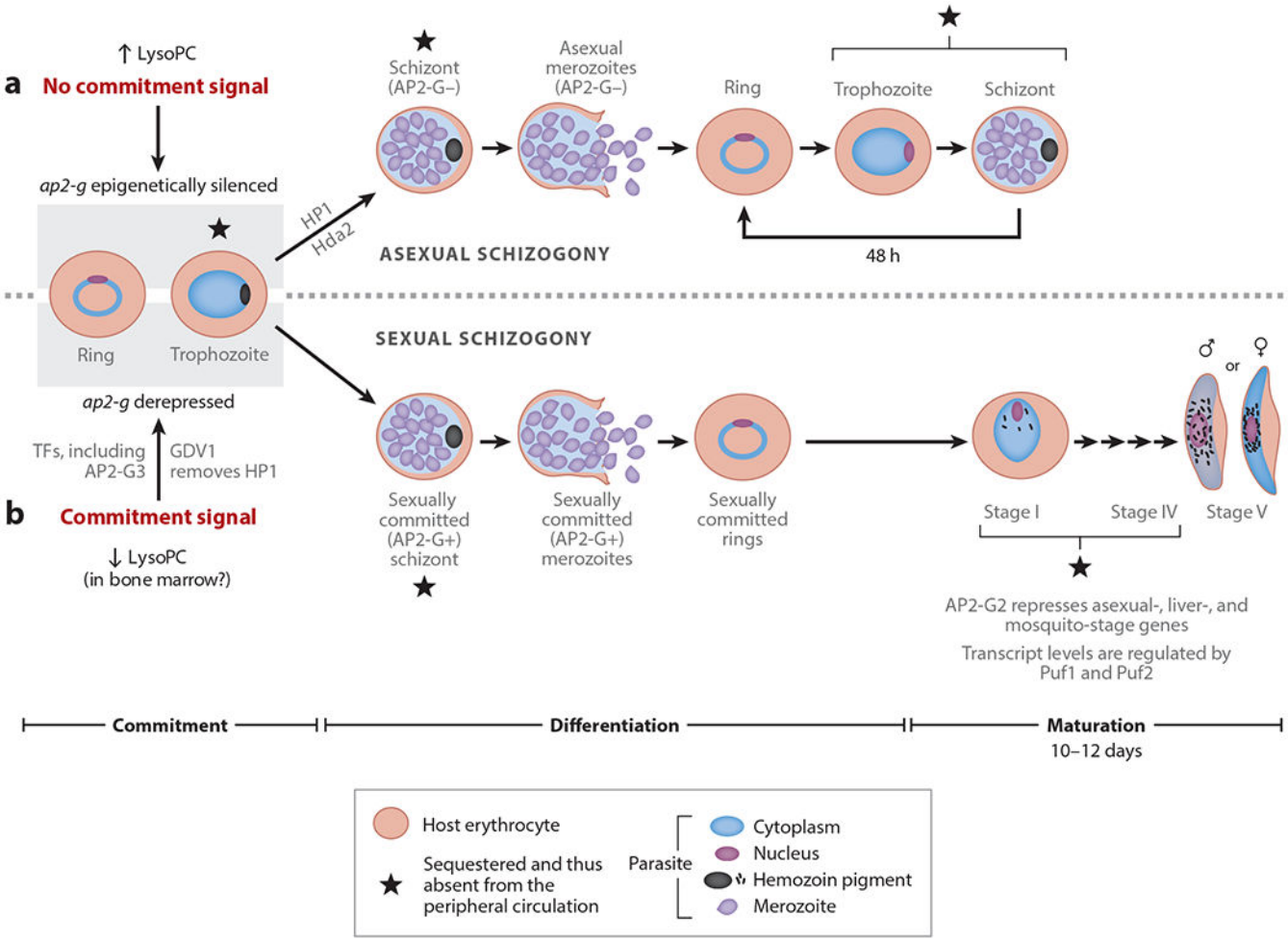

Figure 2.

Model for the regulation of commitment and gametocytogenesis. In the presence of lysophosphatidylcholine (LysoPC), the *ap2-g* locus is epigenetically silenced through the effects of HP1 and Hda2, and cells continue to develop asexually. In the presence of lower levels of LysoPC, parasites are more likely to commit to gametocytogenesis. This process involves the removal of HP1 from the *ap2-g* locus (likely by GDV1), and transcription of *ap2-g* (likely by AP2-G3). Downstream of AP2-G, other epigenetic and transcriptional regulators are expressed, thus continuing the gametocyte transcriptional program while AP2-G2 simultaneously represses asexual-, liver-, and mosquito-stage genes. RNA-binding proteins such as Puf1 and Puf2 are important in modulating transcript levels. Adapted with permission from Reference 34.

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Table 1

Comparison of gametocytogenesis in the four most commonly studied human and rodent *Plasmodium* species (29, 81)

	Human parasites		Rodent parasites
	P. falciparum	***P. vivax***	***P. berghei/P. yoelii***
Liver stage			
Maturation time	144–168h	192 h	50 h
Erythrocytic stage			
Maturation time	48 h	48 h	24 h
Gametocytogenesis			
Maturation time	10–12 days	48 h	24 h
Morphology	Five stages defined by distinct shape changes (Figure 1) I. Spherical II. Half moon III. Spindle with blunt ends IV. Spindle with pointed ends V. Falciform (sickle shaped)	Maintain spherical shape as they grow	
Location	Stages I–IV sequester; stage V circulates in peripheral blood	Circulate in peripheral blood	
Mosquito stage			
Maturation time	Gametogenesis, <5 min; exflagellation, ~ 10–30 min; fertilization, <1 h; ookinete formation, ~24 h; sporozoite production in oocysts, 10–12 days		

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Table 2

Possible regulators of gene regulation during commitment and gametocytogenesis[a]

Gene	Evidence	Other notes
Epigenetic factors		
ISWI (PF3D7_0624600/PFF1185w)	Upregulated in AP2-G$^+$ cells (both AP2-G-DD and NF54) (60) and upregulated in response to LysoPC depletion (8)	Localizes to central area of nucleus (79)
LSD2 (PF3D7_0801900/PF08_0133)	Upregulated in AP2-G$^+$ cells (both AP2-G-DD and NF54) (60)	
Gametocyte development 1/Gdv1 (PF3D7_0935400/PFI1710w)	A naturally occurring deletion leads to loss of gametocyte formation, and overexpression leads to increased gametocytogenesis (23, 24)	
Histone deacetylase 2/Hda2 (PF3D7_1008000/PF10_0078)	Knockdown leads to derepression of the *ap2-g* locus and increased commitment (18)	
SNF2L (PF3D7_1104200/PF11_0053)	Upregulated in AP2-G$^+$ cells (both AP2-G-DD and NF54) (60) and upregulated in response to LysoPC depletion (8)	
Heterochromatin protein 1/HP1 (PF3D7_1220900/PFL1005c)	HP1 is enriched at the *ap2-g* locus (27, 28), and knockdown leads to derepression of the locus and increased commitment (6)	
Histone deacetylase 1/Hda1 (PF3D7_1472200/PF14_0690)	Upregulated in AP2-G$^+$ cells (both AP2-G-DD and NF54) (60)	
Transcription factors		
SIP2 (PF3D7_0604100/PFF0200c)	Upregulated in AP2-G$^+$ cells (AP2-G-DD only) (60) and transcript stabilized in gametocytes (57)	Involved in heterochromatin formation (26)
PF3D7_0613800/PFF0670w	Upregulated in AP2-G$^+$ cells (NF54 only) (60) and upregulated in response to LysoPC depletion (8)	
PF3D7_1139300/PF11_0404	Upregulated in AP2-G$^+$ cells (both AP2-G-DD and NF54) (60)	
PF3D7_1143100/PF11_0442 (PbAP2-O)	Upregulated/stabilized in gametocytes (45, 57)	Disruption results in strongly reduced numbers of ookinetes (*P. berghei*) (52, 87)
PF3D7_1222400/PFL1075w	Upregulated in AP2-G$^+$ cells (both AP2-G-DD and NF54) (60) and upregulated in response to LysoPC depletion (8)	
PF3D7_1222600/PFL1085w (PfAP2-G, PbAP2-G, PyAP2-G)	Naturally occurring mutations, disruption, or knockdown prevents gametocyte formation (37, 71, 88); overexpression leads to increased gametocyte commitment (39)	
PF3D7_1317200/PF13_0097 (PyAP2-G3)	Insertion of the piggyBac transposon in the gene (*P. falciparum*) or deletion (*P. yoelii*) leads to reduced gametocyte formation (34, 88)	
PF3D7_1408200/PF14_0079(PbAP2-G2, PyAP2-G2)	Disruption leads to greatly reduced gametocyte numbers (in *P. berghei* and *P. yoelii*) (71, 85, 88)	
PF3D7_1429200/PF14_0271	Upregulated in gametocytes (45)	Disruption is possible, but no phenotype has been described (46)
AP2-exp (PF3D7_1466400/ PF14_0633, PbAP2-SP)	Upregulated in AP2-G$^+$ cells (AP2-G-DD only) (60)	Involved in regulating clonally variant gene families (*P. falciparum*) (48), required for sporozoite formation (*P. berghei*) (52, 86)
Posttranscriptional regulators		
Puf2 (PF3D7_0417100/PFD0825c)	Disruption leads to increased numbers of gametocytes, as Puf2 translationally represses gametocyte transcripts (50, 51)	
Puf1 (PF3D7_0518700/PFE0935c)	Disruption leads to decreased numbers of mature gametocytes (66)	

Gene	Evidence	Other notes
Alba4(PF3D7_1347500/ MAL13P1.237)	Disruption leads to fewer centers of movement (i.e., less exflagellation) (in *P. yoelii*) (53)	

Abbreviation: LysoPC, lysophosphatidylcholine.

[a]Genes shown either have a gametocyte phenotype upon depletion or disruption or are upregulated in AP2-G-expressing cells or gametocytes (60). Other studies that indirectly influence expression of *ap2-g* also identify possible regulators (6, 8, 18), but because these reports are confounded by AP2-G-independent effects they are not shown here.

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