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Review Article

ApiAP2 Gene-Network Regulates Gametocytogenesis in *Plasmodium* Parasites

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Malaria is a mosquito-borne infectious disease, caused by unicellular Apicomplexan protozoa of the genus *Plasmodium*. The sexual stage of *Plasmodium* is one of the most fascinating aspects of the *Plasmodium* life cycle, yet relatively less explored until now. The production of sexually fit gametocytes through gametocytogenesis is essential to the transmission of the *Plasmodium* parasite into an anopheline mosquito vector. Understanding how gametocytogenesis is regulated promotes the identification of novel drug targets and also the development of transmission-blocking vaccines that would help reduce the disease burden in endemic areas. Transcriptional regulation in *Plasmodium* parasites is primarily controlled by a family of twenty-seven Apicomplexan Apetela 2 (ApiAP2) genes which act in a cascade to enable the parasite to progress through its asexual replication as well as gametocytogenesis. Here, we review the latest progress made on members of the ApiAP2 family characterized as key players of the transcriptional machinery of gametocytes. Further, we will highlight the transcriptional regulation network of ApiAP2 genes at each stage of gametocytogenesis.

1. Introduction

Malaria is considered as one of the diseases that has existed for centuries; it remains one of the devastating infectious diseases ever known. Its persistence till now is a reflection of the resilience of its etiological agent—*Plasmodium*—to crucially maintain transmission between mosquito vector and vertebrate hosts. The *Plasmodium* parasite has resisted eradication attempts in sub-Saharan Africa and persists in South-East Asia, Eastern Mediterranean, and the Americas [1]. From the latest world malaria report, a global estimate shows that there are about 241 million cases of malaria, and it accounts for an estimated 627,000 deaths worldwide [2].

The life cycle of *Plasmodium* is characterized by the transition of morphologically, metabolically, and molecularly unique life forms within a vertebrate host and anopheline mosquito vector. As shown in Figure 1, during a blood meal, a female anopheline mosquito inoculates sporozoites into the blood stream, which travel to the liver and undergo asexual replication to produce pockets of schizonts containing thousands of merozoites [3]. The schizonts rupture to release merozoites that enter the systemic circulation and subsequently invade red blood cells marking the initiation of an intraerythrocytic lytic cycle. The intraerythrocyte parasite forms are responsible for most malaria-associated pathologies. Within the erythrocytes, the parasites undergo asexual morphological transformations: early ring, trophozoite, and schizont. The schizonts eventually produce new asexual merozoites to continue invading new erythrocytes. Alternatively, the asexually replicating intraerythrocytic parasite forms differentiate into nonreplicative sexual gametocytes. The transition of intraerythrocytic parasites to gametocyte (gametocytogenesis) truncates the replication of the parasite within the vertebrate host; however, this

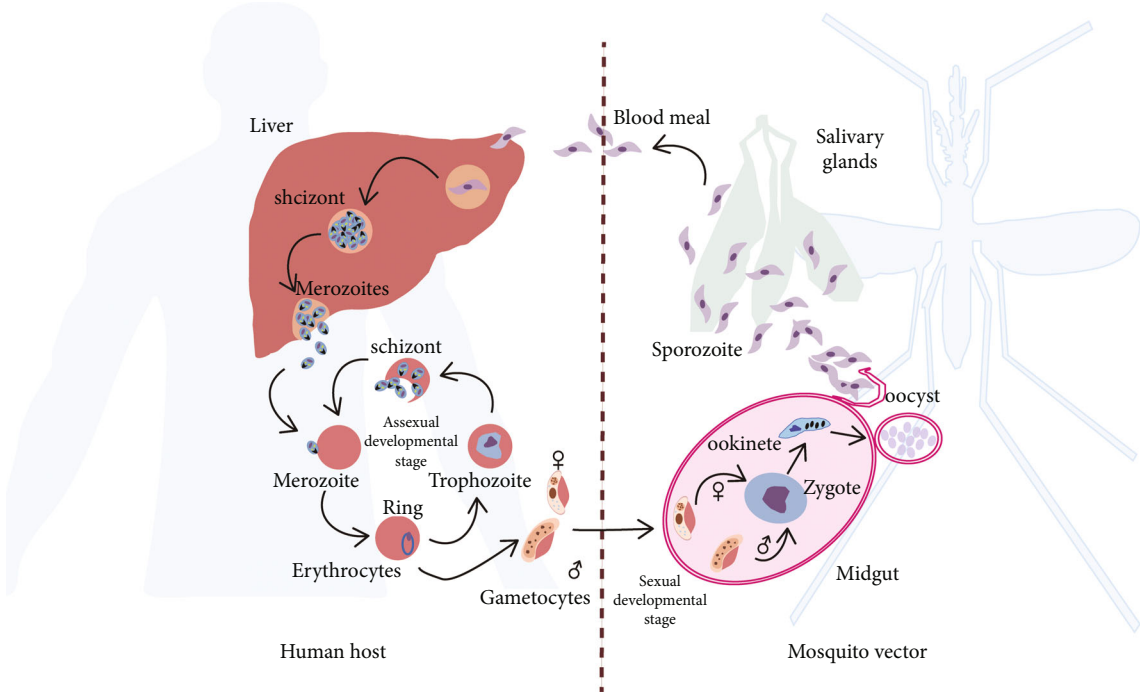


Figure 1: The complete *Plasmodium* life cycle between human host and mosquito vector.

process is crucial for transmission into a mosquito vector [4]. Only a limited proportion of the intraerythrocytic parasites complete the asexual-sexual switch [5–7]. For instance, less than 10% of *Plasmodium falciparum* (*P. falciparum*) intraerythrocytic parasites successfully differentiate into gametocytes in each 48 h cycle [5].

The search to uncover transcriptional regulators of gametocytogenesis is now a domain of intense focus due to their attractive prospects of serving as direct targets of transmission-blocking vaccines and drugs. A family of widely conserved DNA-binding proteins, Apicomplexan Apetela 2 (ApiAP2), have emerged as the key developmental regulators of transcription in *Plasmodium* species [8]. The ApiAP2 genes are homolog to AP2/ERF transcriptional factors from plant lineage and structurally possess one to three DNA-binding AP2 domains [9, 10]. To date, a total of 27 annotated ApiAP2 genes have been described in *P. falciparum* with syntenic orthologs in *Plasmodium berghei* (*P. berghei*) and *Plasmodium yoelii* (*P. yoelii*) (Table 1). Of interest, a subset of ApiAP2 genes, comprising AP2-G, AP2-G2, AP2-G3, and AP2-G5, are particularly integral to the transcriptional machinery of gametocytogenesis among *Plasmodium* species [11, 12]. Functional genomic studies show that the transcriptional regulatory footprints of ApiAP2 genes putatively cover different stages of the entire life cycle of *Plasmodium* species (Table 1). Indeed, investigations into the transcriptional regulation of *Plasmodium* genes by ApiAP2 transcription factors have developed significantly over the last few years making it appropriate for a review. Here, we review the latest work on members of the ApiAP2 family that are essential to the transcriptional machinery of gametocytes. We will cover their involvement in each stage of gametocytogenesis spanning from sexual commitment to sexual maturation and highlight the ApiAP2 gene regulatory network that governs gametocytogenesis.

2. Gametocytogenesis of *Plasmodium* Species

Gametocytogenesis of *Plasmodium* species is a developmental pathway that describes the conversion of in-host erythrocytic parasite forms into sexually fit gametocytes [13], which can be broadly viewed as a three-step process: sexual commitment, sexual conversion, and sexual maturation. Sexual commitment and sexual conversion constitute the initial steps of gametocytogenesis. These two processes are distinct but sequential [14]. Sexual commitment describes parasite forms earmarked to undergo sexual conversion at a later point [14]. In *P. falciparum*, sexually committed parasites are classically characterized by the expression of AP2-G protein marker. At this point, committed sexual rings appear indistinguishable from other asexual intraerythrocytic parasite forms. Sexual conversion ensues sexual commitment. The conversion process is characterized by the stabilization of AP2-G expression and also the expression of GEXP5 [14] via a mechanism independent of AP2-G [15]. There are two known routes of sexual conversion: next cycle conversion (NCC) and same cycle conversion (SCC). Next, cycle conversion (NCC, the conventional route) explains that the asexual-sexual switch occurs before schizogony, and as such, all merozoites released by a single schizont share the same developmental fate by developing into sexual gametocytes or asexual parasites and never both [16, 17]. A later study demonstrated the formation of mixed sexual-asexual plaques where a fraction of the asexual parasite forms are directly converted to sexual forms without going through the next phase of asexual replication [18]. The formation

Table 1: The ApiAP2 genes in *Plasmodium* species.

ApiAP2 encoding gene				Stage(s) of impact	Putative role(s) in *Plasmodium* species		
P. falciparum	Syntenic ortholog in *P. berghei*	Syntenic ortholog in *P. yoelii*	Name		*P. falciparum*	*P. berghei*	*P. yoelii*
PF3D7_0404100	PBANKA_1001800	PY17X_1003200	AP2-SP2	Oocyte-sporozoites	n.a	(i) Enhance production of early sporozoite [75]	(i) Enhance production of early sporozoite [60]
PF3D7_0420300	PBANKA_0521700	PY17X_0523100			n.a		(i) No observed functional role in the entire life cycle [60]
PF3D7_0516800	PBANKA_1231600	PY17X_1235000	AP2-O2	Zygote-ookinete	n.a	(i) Enhance production of infective ookinete [75]	(i) Enhance production of oocyst [60] (ii) Enhance production of salivary gland sporozoites [60]
PF3D7_0604100	PBANKA_0102900	PY17X_0104500	SIP2	Schizont	(i) Repress var gene [91] (ii) Formation of heterochromatin and maintaining genome integrity [91]	n.a	n.a
PF3D7_0611200	PBANKA_0109500	PY17X_0111100			n.a	(i) Mediate host immune evasion [92]	n.a
PF3D7_0613800	PBANKA_0112100	PY17X_0113700			n.a	n.a	n.a
PF3D7_0622900	PBANKA_1121800	PY17X_1123200	AP2-Tel/SP3	Oocyte-sporozoites	(i) Maintain telomerase mechanisms [93]	(i) Production of mature/infective sporozoite [75]	(i) Production of matured/infective salivary gland sporozoites [60]
PF3D7_0730300	PBANKA_0214400	PY17X_0215800	AP2-L	Liver stage sporozoites	n.a	(i) Enhance infectivity and maturation of liver-stage sporozoites [94]	n.a
PF3D7_0802100	PBANKA_1228100	PY17X_1231600			n.a	n.a	n.a
PF3D7_0934400	PBANKA_0835200	PY17X_0838600			n.a	n.a	n.a
PF3D7_1007700	PBANKA_1205900	PY17X_1209100	AP2-I		(i) Regulate RBC invasion genes [95]	n.a	n.a
PF3D7_1107800	PBANKA_0939100	PY17X_0941600					
PF3D7_1115500	PBANKA_0932300	PY17X_0934300			n.a	n.a	n.a
PF3D7_1139300	PBANKA_0909600	PY17X_0911000			n.a	n.a	n.a
PF3D7_1143100	PBANKA_0905900	PY17X_0907300	AP2-O	Zygote, ookinete, blood-stage, gametocyte	(i) Regulate Var gene switching in intraerythrocytic asexual parasite forms [82] (ii) Promote recruitment of HP1 and indirectly repress gametocyte-associated genes [82] (iii) Increase production of oocytes [82]	(i) Production of ookinete [75, 96, 97] (ii) Promote mosquito immunity evasion [97]	(i) Production of ookinete [60]
PF3D7_1222400	n.a.	n.a.	AP2-G4		(i) Contribute to sexual commitment but its role is not well-defined [66]	n.a	n.a

Table 1: Continued.

ApiAP2 encoding gene				Stage(s) of impact	Putative role(s) in *Plasmodium* species		
P. falciparum	Syntenic ortholog in *P. berghei*	Syntenic ortholog in *P. yoelii*	Name		*P. falciparum*	*P. berghei*	*P. yoelii*
PF3D7_1222600	PBANKA_1437500	PY17X_1440000	AP2-G	Asexual-gametocyte	(i) Initiate sexual commitment and regulate early gametocyte development [26] (ii) Regulate RBC invasion gene [24]	(i) Responsible for sexual differentiation [98]	(i) Initiate sexual commitment [60] (ii) Regulate early gametocyte development [60]
PF3D7_1239200	PBANKA_1453700	PY17X_1456200			n.a	n.a	n.a
PF3D7_1305200	PBANKA_1403700	PY17X_1405400			n.a	n.a	n.a
PF3D7_1317200	PBANKA_1415700	PY17X_1417400	AP2-G3/FG	Gametocyte	(i) Transmits cytoplasmic signals into the nucleus to initiate *ap2-g* transcription [28]	(i) Enhance maturation of female gametocytes [45]	(i) Transmits cytoplasmic signals into the nucleus to initiate *ap2-g* transcription [60] (ii) Enhance maturation of both male and female gametocytes [60]
PF3D7_1342900	PBANKA_1356000	PY17X_1361700			n.a	n.a	n.a
PF3D7_1350900	PBANKA_1363700	PY17X_1369400	AP2-O4	Zygote-ookinete	n.a	(i) Production of infective ookinete [75]	(ii) Enhance early oocyst development [60]
PF3D7_1408200	PBANKA_1034300	PY17X_1036700	AP2-G2	Asexual-gametocyte	(i) Enhance the production and maturation of gametocytes, described in detail in AP2-G2 part of the text	(i) Enhance maturation of gametocytes [25] (ii) Repress ookinete, sporozoite, liver, and merozoite stage genes [68, 75]	(i) Enhance maturation of gametocytes [60]
PF3D7_1429200	PBANKA_1015500	PY17X_1017000	AP2-O3	Zygote-ookinete	n.a	(i) Enhance the production and maturation of ookinete [75] (ii) Repress microgametocyte genes at the ookinete stage [75] (iii) Involved in zygote metabolism [75]	(i) Enhance the production and maturation of ookinete [60] (ii) Enhance transcription of genes that promote the expression of ookinete development genes [60] (iii) Enhance transcription of female-specific genes [44] (iv) Repress male-specific genes [44]
PF3D7_1449500	PBANKA_1313200	PY17X_1317000	AP2-O5	Zygote-ookinete	n.a	(i) Enhance the production of motile ookinete [75]	(i) Enhance the production of motile ookinete [60]
PF3D7_1456000	PBANKA_1319700	PY17X_1323500	AP2-HC		(i) No observed functional role in gametocytes [83]	(i) No observed functional role in entire life cycle [75]	(i) No observed functional role in entire life cycle [60]
PF3D7_1466400	PBANKA_1329800	PY17X_1334500	AP2-SP/EXP	Asexual-gametocyte	(i) Involved in the proliferation of intraerythrocytic forms [99] (ii) Repress var genes [99] (iii) Enhance the production of sporozoite [100]	(i) Enhance the production of sporozoite [75] (ii) Negative regulator of female-specific genes [75]	n.a

Notes: n.a: not available.

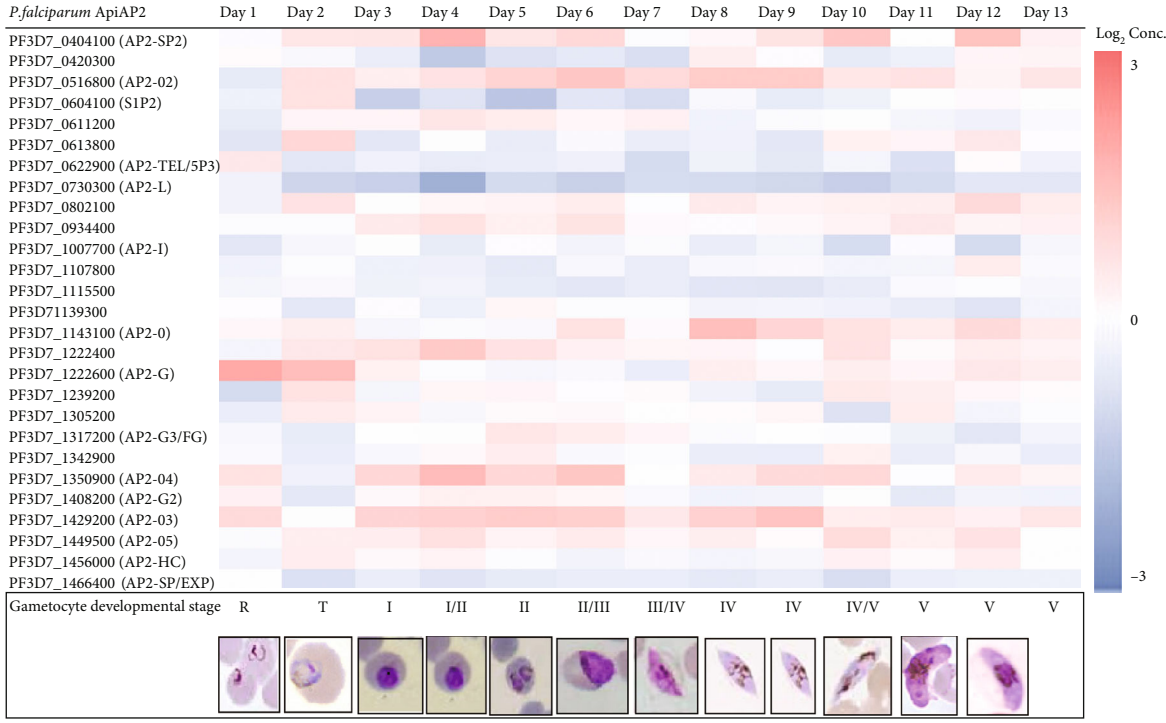


FIGURE 2: Dynamic expression pattern of 27 *Pf*ApiAP2 genes during gametocytogenesis over a 13-day *in vivo* culture. The map shows time-course expression from the committed ring stage to stage V. Red represents regions where the ApiAP2 gene is highly expressed ($Log_2Conc > 0$), and blue represents regions of low expression ($Log_2Conc < 0$). The white region shows regions where Log_2 concentration expression is equal to or approximately equal to zero ($Log_2 = 0$). A micrograph of the gametocyte developmental stages that correlate with the days and dynamic expression of the genes is shown. Ring stage: R; trophozoite stage: T; gametocytes mature through five morphologically distinct stages I-V. The transcriptomic data were extracted from Biljon et al. [27] for the graphical illustration. The images of the gametocyte developing stages were adopted from the Centers for Disease Control and Prevention [89] and Wadi et al. [90].

of sexual-asexual mixed parasite plaques occurs via same cycle conversion (SCC) [18].

A successful sexual conversion is immediately followed by the third step, sexual maturation. Noteworthy, there are durational differences in sexual maturation among *Plasmodium* species. For instance, gametocyte development in *P. falciparum* takes 8-12 days (Figure 2), whereas that of *P. berghei* usually lasts less than 2 days [19, 20]. In *P. falciparum,* successfully converted gametocytes mature through five morphologically distinct stages: stages I-V. Early gametocytes (Stages I and IIa) appear indistinguishable from each other, which are characterized by the emergence of a tri-laminar membrane structure, a pellicular membrane complex from the gametocyte plasmalemma resulting in gross morphological restructuring [13, 21]. Morphological transformation into crescent gametocyte begins at late stage IIb [22]. At this stage, the parasite appears to assume a D-shape and occupies about half the volume of the red blood cell [23]. During stages III-IV, subpellicular membranes are arranged such that it gives an elongated shape with rounded ends [23]. The subpellicular microtubules disintegrate at stage V [22]. At this stage, the gametocyte assumes a characteristic crescent shape or sickle shape [23]. Sexual dimorphism/divergence into female gametocyte (macrogametocyte) or male gametocyte (microgametocyte) is visible between stages IV-V. Sexual forms of Plasmodium vivax, Plasmodium berghei and Plasmodium yoelii asume a spherical shape throughout maturation.

3. Regulation of Gametocytogenesis by ApiAP2 Genes

So far, as shown in Table 1 and Figure 2, eight ApiAP2 genes including AP2-G, AP2-G2, AP2-G3/FG, AP2-G4, AP2-G5, AP2-O, AP2-O3, and AP2-SP/EXP are shown to be involved in gametocytogenesis of *Plasmodium* parasites of which five ApiAP2 genes including AP2-G, AP2-G2, AP2-G3/FG, AP2-G5, and AP2-O3 have been extensively characterized to putatively play important roles in *Plasmodium* parasites. Below, we provide details of their phenotype(s)/role(s) in gametocytogenesis of *Plasmodium* parasites.

3.1. Regulation of Gametocytogenesis by AP2-G

3.1.1. AP2-G Phenotypes. In *P. falciparum*, the binding site of *Pf*AP2-G is identified across three parasite stages: schizont, ring , and stage I gametocytes [24]. Electrophoretic mobility assay analysis reveals the binding motif of *Pf*AP2-G as G × GTAC×G [25]. Transcriptional control by *Pf*AP2-G is achieved by binding to (G/G) TAC and its reverse GTA (C/C) motifs, which were found upstream of hundreds of putative regulons including *ap2-g* itself [24, 25]. In *P. berghei*, ChIP-seq analysis showed that the binding site of *Pb*AP2-G is constituted of a pair of six-base motifs, GTACTT and GTACAC [11].

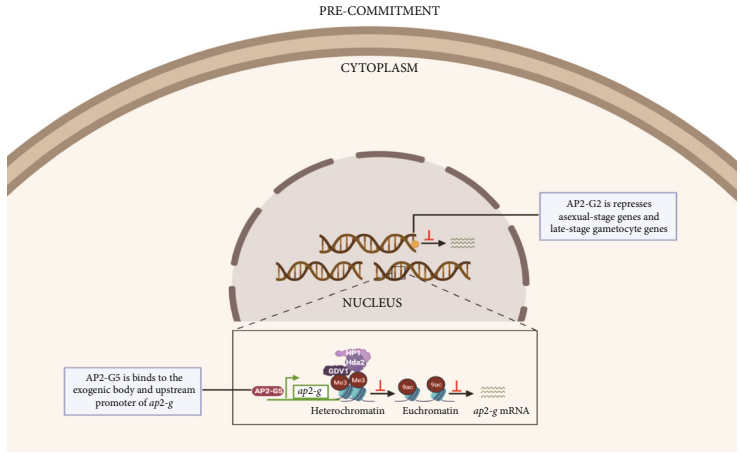
Gametocytogenesis thrives under the control of different but functionally linked transcription factors. Of these, AP2-

G is a central factor that plays an indispensable role in ushering asexual *Plasmodium* parasites into the sexual cycle. AP2-G is dubbed the "master regulator" of gametocytogenesis. The central role of AP2-G required for the asexual-sexual switch was first shown in *P. falciparum* and *P. berghei* which harbored mutations in *ap2-g* and had lost the capacity to produce gametocytes [25, 26]. Recently, ChIP-seq analysis of an*in vitro* cultured transgenic *P. berghei* has substantiated the essentiality of AP2-G in gametocyte production and provided insight into its underpinning mechanism. This study posited that *Pb*AP2-G is located at the summit of a gametocytogenesis-specific transcriptional cascade encompassing several ApiAP2 transcriptional factors [11]. The downstream transcription factors include AP2-G2, AP2-FG, and AP2-O3 [11]. As such, the expression of a fully functional AP2-G switches on the gametocytogenesis-specific transcriptional cascade [24, 25, 27] and thereby activates the regulatory control by downstream ApiAP2 transcription factors culminating in the production of a high number of gametocytes. On the opposites, AP2-G inactivation or disruption generates a negative ripple effect on downstream gametocyte-specific transcription factors resulting in a complete loss of capacity to produce gametocytes. Interestingly, the role of AP2-G as the "molecular switch" to gametocytogenesis is evolutionarily conserved across most *Plasmodium* species, highlighting the essentiality of AP2-G to the process (Table 1).

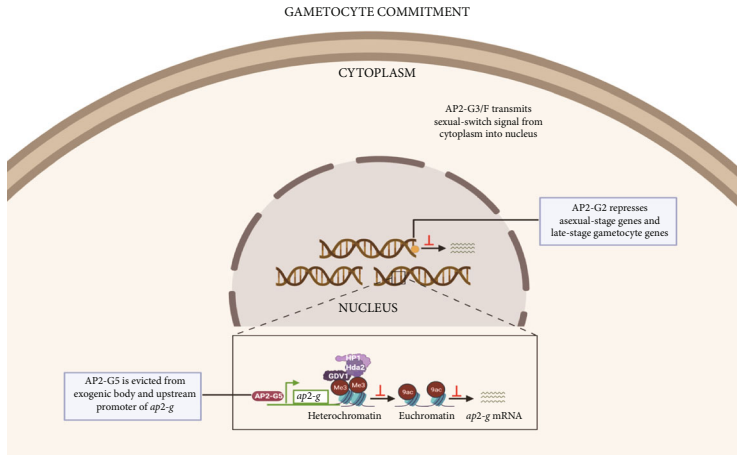
As with all essential genes, stabilization of their expression is crucial to optimizing their function. Maintaining a high-steady concentration of mRNA transcript allows for an enhanced gene expression. The stabilization of AP2-G expression is thought to be in part promoted epigenetically by gametocyte development 1 (GDV1) and AP2-G in a positive autoregulatory feedback loop (Figure 3(c)) [28]. In sexually committed parasites, a stable expression of AP2-G not only facilitates the establishment of a gametocytogenesis-specific transcriptional program but also determines the route via which the parasite will be sexually converted. Thus, the time point at which AP2-G expression is successfully stabilized is critical. At one end, stabilization of *Pf*AP2-G in early rings facilitates the conversion of committed parasites via the SCC pathway [29]. On the other end, stabilization of *Pf*AP2-G in early rings favours the NCC pathway [18]. Intriguingly, the transcriptome analysis of gametocytes derived from these two pathways is demonstrated as light difference in their gene expression pattern. For instance, in *P. falciparum*, there were higher transcripts of *pfg.14.748*, *epf1*, and *pf3d71476600* levels in gametocytes converted via the NCC pathway than the SCC pathway [14]. Furthermore, ChIP-seq analysis has revealed a difference in *Pf*AP2-G occupancies between stage I gametocytes produced from SCC and NCC pathways [24]. Specifically, the *Pf*AP2-G binding site appeared higher on AP2-G target genes of NCC-derived gametocytes compared with SCC-derived gametocytes [24]. Against this, *Pf*AP2-G may induce different phenotypes from early gametocytes obtained via these two routes, but further studies are required to validate this posit.

Once asexual parasites have been successfully converted into gametocytes, AP2-G continues to regulate the maturation of early gametocytes. Evidently, in *P. falciparum*, ChIP-seq analysis revealed that the AP2-G binding site is highly enriched upstream of early gametocyte gene markers. These include *Pf*g16, *Pf*g27/25, etramp10.3, *Pf*g14.744, *Pf*g14.745, and *Pf*g14.748 [24, 30]. The expression of these known genes governs the maturation of early gametocytes [31–34].

Along the course of maturation, developing gametocytes diverge into specialized haploid male and female sexes. The fact that a haploid *Plasmodium* parasite gives rise to a haploid male and female sexual progenies suggests that sex determination does not arise from different genetic content but through differential gene expression [35]. Nonetheless, the genetic determinants underpinning this event are obscure. As established, sexual commitment precedes or is concurrent with sex determination [36] and as such the prominent role of AP2-G in initiating sexual commitment somewhat predicts that AP2-G may as well be involved in sex determination. Until recently, it remained a puzzle how AP2-G directs the differential expression of male and female-specific genes from a haploid precursor, but now, studies have begun to redress this process. Emerging evidence suggests that AP2-G orchestrates a sex-determining cascade that drives the bifurcation of gametocytes into distinct male and female sexes [37]. Earlier, a recombinant gene-based AP2-G overexpression system was used to demonstrate that overexpression of *Pb*AP2-G coincides with the upregulation of zinc finger proteins, RNA binding proteins, and helicases [38], which are usually encoded by canonical sex-specific genes. Consistent with these findings, a global single-cell transcriptome analysis of mutagenized *P. berghei* confirmed that zinc finger proteins [PBANKA_0413400 (md3), PBANKA_0828000 (gd1) and PBANKA_1435200 (fd4)], RNA binding proteins [PBANKA_0716500 (md5), PBANKA_1454800 (fd1)], OST-HTS-associated domain protein [PBANKA_1302700 (md1)], AT-rich interactive domain-containing protein [PBANKA_0102400(md4)], ACDC domain-containing protein [PBANKA_1418100(fd3)], and two conserved proteins of unknown function [PBANKA_1447900 (md2), PBANKA_0902300 (fd2)] are crucial early sex-determining markers, putatively activated downstream by AP2-G [37]. Using uniform manifold approximation and projection plot, further evidence from Russell et al. [37] suggested that sexually committed parasites initially follow a common transcriptional pathway but bifurcate into male and female-specific transcriptional pathways of which the peaking of AP2-G was identified as the common diverging point [37]. Immediate to the induction of sex-determining cascade by AP2-G, md1, and md2 mediate upstream divergence of male gametocytes whereas gd1 and md3 mediate the divergence of female gametocytes. At one diverged end, male gametocytes undergo maturation by the expression of known male-specific proteins, such as gd1, md4 [37], alpha-tubulin 2 [39], P230p [40], and P48/45 [41]. At the other end, female gametocytes mature under the expression of female-specific proteins, such as fd1, fd2 [37], DOZI [42], p25, p28 [43], AP2-O3 [44], AP2-G3/FG [45], NEK2, NEK4 [46], CCP1, and CCP3 [47]. Once the sex-determining transcriptional cascade is activated, AP2-G likely directly augments the downstream transcriptional



(a)



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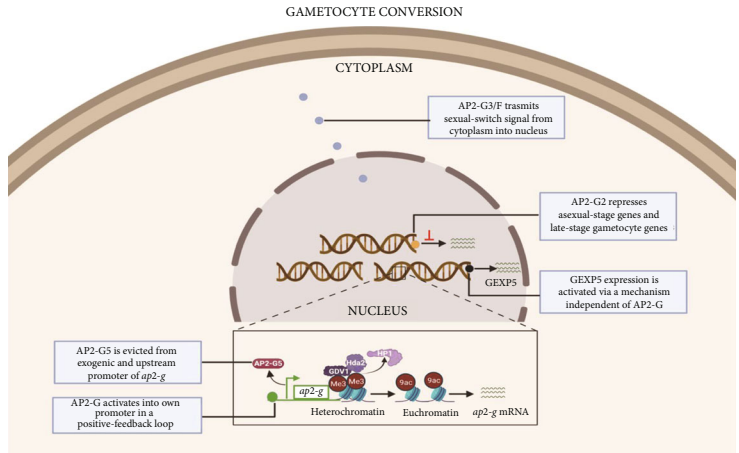

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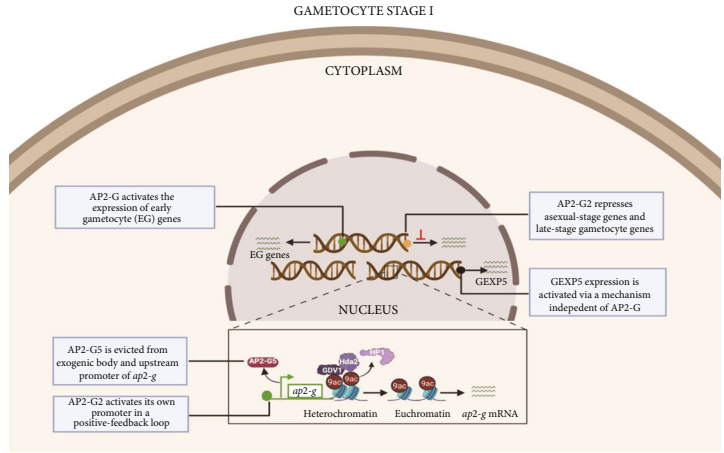

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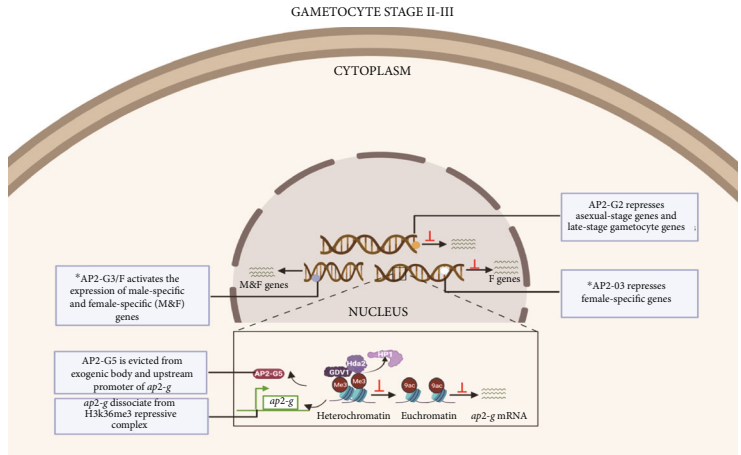

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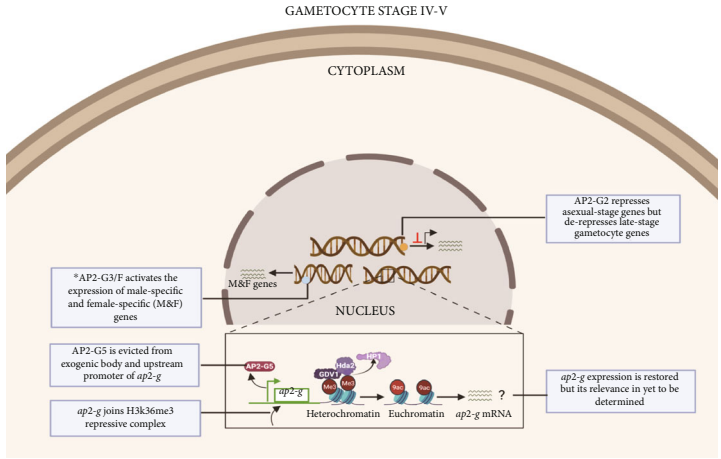

(f)

Figure 3: ApiAP2 gene-network regulates gametocytogenesis in *Plasmodium* species. (a) Precommitment AP2-G2 represses sexual stage genes preventing the initiation of gametocytogenesis. AP2-G5 binds to *ap2-g* and directly inhibits its expression. (b) Sexual commitment AP2-G3/FG transmits cytoplasmic signals of sexual switch into the nucleus. AP2-G5 is evicted from the exogenic body and upstream promoter of *ap2-g*. HP1 is also evicted from the H3K36me3 repressive complex and thus initiates gametocyte commitment. (c) Sexual conversion AP2-G binds to its own promoter and increases *ap2-g* expression which drives the conversion of sexual commitment rings into early-stage gametocytes. Time-point stabilization of *ap2-g* expression determines the route of sexual conversion; next cycle conversion (NCC) or same cycle conversion (SCC) pathways (not shown in the diagram, but described in detail in the text). GEXP5 is expressed concomitantly with AP2-G to regulate the sexual conversion process. (d, f) Sexual maturation. During stage I, AP2-G increases the expression of early-stage (EG) gametocyte genes. AP2-O3 regulates the maturation of female gametocytes between stages II-III. AP2-G2 putatively represses the expression of late gametocyte-stage genes to prevent their premature expression but is derepressed at stages IV-V. AP2-G2/FG controls late-stage sexual dimorphism of female gametocytes (in *P. berghei*) or the maturation of both male and female gametocytes from stage II down through to stage V. "∗" indicates possible different phenotypes among different *Plasmodium* species; HP1: heterochromatin protein 1; Hda2: histone deacetylase 2; H3K9me3: Histone 3 lysine 9 trimethylation; GDV1: gametocyte development 1. Schematic created with Biorender.com.

control of sex-specific genes by other ApiAP2 transcription factors. Evidently, ChIP-seq analysis has shown that the *Pb*AP2-G binding site is highly enriched upstream of well-established sex-specific AP2-related transcription factors including AP2-FG and AP2-O3, which acts downstream of AP2-G [37]. Perhaps this provides a glimpse into the role of the *Pf*AP2-G beyond stage I (Figure 2). However, whether the sex-determining cascade proposed by Russell et al. [37] is adjusted or influenced by the genetic diversity of *Plasmodium* species has not been tested. Hence, it will be exciting to investigate the sex-determining transcriptional cascade in other *Plasmodium* species. Further, investigations into the involvement of epigenetic control at each point of the sex-determining cascade will be crucial to a holistic understanding of the process.

The asexual-sexual switch is marked by a repertoire of transcriptional changes, and this coincides with changes in host-immunological response [48–52]. As such, the sexually developing parasites are constantly faced with the prospect of elimination by host immune responses. In light of this, concurrent tight regulation of gametocytogenesis and surface antigen expression is crucial to ensure the survival of gametocytes. The highly polymorphic var genes encode the major antigenic/virulence protein, *Pf*EMP1, which represents the prime immune targets of the human host [53]. As described previously, *Plasmodium* parasites adopt a facultative heterochromatin-mediated control of var genes, where only a singular variant antigenic gene is expressed at any given time to ensure a variegated gene expression, which is crucial to minimize host immune recognition [54]. *Ap2-g* locus is proximal to var genes, and both share a close association with HP1 and H3K9me3 repression domain suggesting the expression of both genes is under similar heterochromatin-mediated control [55]. This signifies a potential link between sexual commitment and variegated expression of *Pf*EMP1. One postulate suggested that the expression of AP2-G is mutually exclusive with the expression of var genes [45]. This is argued for by the fact that AP2-G and *Pf*EMP1 genes are flanked by a common silencing molecule, an insulator-like pairing element (PE), and thus, both are silenced in a similar manner [26, 54]. Nonetheless, this hypothesis is challenged by the assertion that sexual commitment occurs before gametocytogenesis [16, 36, 56], and also, var gene and *ap2-g* are concurrently expressed, at least during the commitment cycle. At the committed trophozoite and schizont stages, *Pfap2-g* significantly interacts with the nearest var gene cluster demonstrating a direct genic interaction [57]. However, this interaction is lost around stages II-III as AP2-G dissociates from the H3K9me3 repressive complex [57]. The importance of this interaction is however unknown. Interestingly, emerging evidence suggests that upstream promoters of var genes that encode *Pf*EMP1 proteins involved in immune evasion and cytoadhesion are associated with AP2-G [24]. This suggests that var genes that encode *Pf*EMP1 proteins are potential direct targets of AP2-G, implying AP2-G possibly influences the variegated expression of *Pf*EMP1. Against

the backdrop that the promoters of var genes play a crucial role in maintaining epigenetic memory, AP2-G is more likely to crosstalk with the promoters of var genes to control the expression of *Pf*EMP1 variants in the mitotic progeny [58, 59]. However, further investigation is needed to fully delineate the relationship between AP2-G expression and variegated expression of *Pf*EMP1 in the context of host immune evasion.

3.1.2. Regulation of AP2-G. Despite the role of AP2-G as the "master switch" to sexual differentiation, the molecular players that direct its transcription remain obscure. Emerging findings point to AP2-G3 as a putative candidate [28]. Among *P. falciparum* and *P. yoelii* parasites, AP2-G3 has been shown to act upstream of *ap2-g* and transduce cytosolic transcriptional signals into the nucleus to enhance the transcription of AP2-G (Figure 3(a)) [28, 60]. This finding is based on the fact that AP2-G3 is expressed earlier during sexual commitment, and it is localized maximally in the cytoplasm compared to the nucleus. Evidently, *ap2-g3* disruption reduces the expression of AP2-G but disruption in *ap2-g* has no bearing on AP2-G3 expression. Aside AP2-G3, AP2-G5 has also been implicated in the transcription regulation of *ap2-g*. A single-cell transcriptome analysis revealed that AP2-G5 is expressed following the expression of AP2-G during sexual commitment [61]. A follow-up study showed that disruption in or deletion of *ap2-g5* binding domain in two *P. falciparum* parasite clones, NF54 and 3D7-G7, correlated with upregulation of AP2-G during sexual commitment, suggesting that AP2-G5 is a repressor of AP2-G. Further investigation using ChIP-seq analysis revealed that *Pf*AP2-G5 directly binds to both the exogenic body and the upstream of *Pfap2-g* and represses its expression, thereby hampering the initiation of gametocyte commitment.

Aside it being transcriptionally regulated, *ap2-g* is also epigenetically regulated. The *ap2-g* promoter is identified with repressive histone marks, including Histone 3 lysine 9 trimethylation (H3K9me3), heterochromatin protein 1 (HP1), and histone deacetylase 2 (Hda2) [54, 62]. H3K9me3 provides a docking site for HP1 [54]. As HP1 binds to methylated H3K9 and sustains repression, Hda2 regulates silencing at the *ap2*-locus [63, 64]. In *P. falciparum*, gametocyte development 1 (GDV1) antisense RNA induces the expression GDV1 which consequently binds to the H3K9me3-HP1-*ap2-g* complex and thus facilitates the eviction of HP1 [65]. Eviction of HP1 induces posttranslational modification of the histone tail via acetylation [29]. This culminates in the conversion of heterochromatin to euchromatin leading to increased expression of AP2-G and thus functionally drives gametocyte commitment (Figure 3(c)). Recently, in trophozoites of *P. falciparum*, AP2-G5 has been shown to alter the GDV1-H3K9me3 interaction by reducing the occupancy of GDV1 via a mechanism independent of HP1 and Hda2 thereby maintaining a heterochromatin state [66]. Interestingly, AP2-G has sufficient power to drive gametocyte commitment even when GDV1 is defective or inhibited. This could result from other unknown auxiliary or complementary players that coregulate the activation of *ap2-g* beside GDV1. This is supported by the evidence that *ap2-g* is not entirely dependent on HP1 and Hda2 and therefore might not necessarily require HP1 eviction to initiate sexual commitment [54]. This is strengthened by the fact that nonhuman *Plasmodium* parasites that lack syntenic GDV1 protein sufficiently undergo sexual commitment, which further suggests the functional redundancy of GDV1 in the sexual commitment process [65]. At the postcommitment stage, AP2-G is silenced by histone 3 lysine 36 dimethylation/tri-methylation (H3K36me2&3) and AP2-G2 once its function is no longer needed [67].

3.2. Regulation of Gametocytogenesis by AP2-G2

3.2.1. Phenotypes of AP2-G2. The study of AP2-G2 phenotypes is currently complicated probably due to its genome-wide targets comprising a wide array of sexual and asexual stage specific genes. However, AP2-G2 is typically recognized as a global transcriptional repressor. In *P. berghei*, AP2-G2 binds to a predicted short five-base nucleotide GTTC (T/C), and its reverse complement (A/G) CAAC is found upstream of about 1500 genes [68]. Aberrant to this, a different study identified AGAA and ACCA as the predictive binding motifs of *Pf*AP2-G2 for approximately 3000 putative genes [12]. Thus, despite their phenotypic similarities, target genes for AP2-G2 are heterogeneous among *P. berghei* and *P. falciparum* [69]. The difference in gene targets could be the consequence of posttranslational modifications that alters the binding specificity of AP2 proteins *in vivo* [70]. For instance, lysine acetylation, which is relatively prevalent in *Pf*AP2 proteins, changes the binding recognition of AP2 proteins [70, 71].

Based on gene targets of *Pb*AP2-G2 obtained through ChIP-sequence analysis, one study speculated that AP2-G2 represses asexual genes required for asexual replication, and thereby ushers asexual parasites into sexual commitment [68]. A shred of different evidence from gametocyte transcriptomics demonstrated that, indeed, *Pf*AP2-G2 represses asexual stage-specific genes including knob-associated histidine protein, hypothetical gene family protein, merozoite surface proteins, and serine repeat antigen 5 [12]. Yet, parallel to the earlier speculation, it appeared that the asexual stage repression effect of AP2-G2 in *P. falciparum* has no discernible impact on the replication rate of asexual stage parasites or sexual commitment [12].

A host of evidence indicates that aberration in or deletion of *ap2-g2* substantially hampers the maturation of gametocytes [68, 72]. Specifically, gametocytes with defective *ap2-g2* seem not to mature beyond stage III [12, 68, 72]. Across three different studies, the expression of crucial late gametocyte markers has been reported to significantly decrease in the absence of AP2-G2 activities [12, 48, 68]. Of these, transcripts of dynein heavy chain, p25, and p28 have been consistently shown to be reduced across these three studies [12, 48, 68]. The disintegration of the gametocyte before stage III could be explained in two folds. First, the premature gametocyte disintegration could be the consequence of the reduced expression of the essential late gametocyte genes which are required to maintain the integrity of matured gametocytes [72–74]. Second, the untimely expression of stage-specific genes in the absence of AP2-G2

repression may underscore the premature disintegration of the gametocytes. If the former hypothesis is true, it remains plausible that AP2-G2 is "bi-functional" acting as an activator (direct or indirect) of critical late gametocyte genes beyond stage III and also a repressor at the other life stages of the *Plasmodium* parasite. Interestingly, alteration in *ap2-g2* of gametocytes already expressing sex-specific genes only resulted in reduced expression of the sex-specific genes and not an abrogation of the maturation process [68]. This somewhat supports the posit that AP2-G2 is a selective activator of a subset of essential sex-specific genes critically needed by gametocytes to develop beyond stage III—which is a deviation from its conventional repression function.

There are conflicting conclusions, nonetheless, about the effect of AP2-G2 on the gametocyte sexual dimorphism in the context of sex ratio. For instance, a gene knockout study in *P. falciparum* has demonstrated a disproportionate inhibition of male gametocyte divergence compared with their female gametocyte counterparts [72]. The skewed repression of male gametocytes production in AP2-G2 knockout mutants reflects the downregulation of *Pf*MDV-1, an essential gene in the production of male gametocytes [72]. Paradox to this, other studies conducted in *P. berghei* observed no effects of AP2-G2 on sex ratio but observed increased expression levels of both male and female sex-specific genes [68, 75]. Possibly, the specific sex-determining effect of AP2-G2 could be species-dependent or it could be dependent on AP2-G2 expression threshold. Hence, further investigation to ascertain the effect of AP2-G2 expression threshold on sexual dimorphism would be crucial.

3.2.2. Regulation of AP2-G2. The understanding of the transcriptional regulation of AP2-G2 is complicated by the heterogeneity of *Plasmodium* species. In one study, the ChIP-seq analysis demonstrated that the upstream of *Pb*AP2-G2 possesses multiple binding motifs of *Pb*AP2-G [11]. Further findings showed that the transcription of *Pbap2-g2* is directly induced by *Pb*AP2-G approximately around the time when sexually committed parasites are being converted into stage I gametocytes [11]. In contrast, RNA-seq and RT-qPCR analysis of schizont with disrupted *Pfap2-g2* showed a reduced expression of *Pf*AP2-G implying that *Pf*AP2-G2 acts upstream of *Pf*AP2-G at the schizont stage [72]. Nonetheless, it is not known whether the reduced expression *of ap2-g* in the schizonts was induced directly or indirectly by AP2-G2. Deductively, it seems that the effect of AP2-G on *ap2-g2* transcription is somewhat dependent on the developmental stage of the *Plasmodium* parasite.

Given the immense role of histone posttranscriptional modifications (hPTM) in epigenetic control of ApiAP2 genes, there is a plausible prospect of their functional link to AP2-G2. Accordingly, recent studies have begun to explore the epigenetic relationship between AP2-G2 and hPTM variants. For instance, it has been demonstrated that AP2-G2 and H3K36me2&3 share 33% common regulons in stages II-III gametocytes [67], suggesting that AP2-G2 and H3K36me2&3 are more likely to form a complex to initiate a coordinated repression control at stages II-III. However, stages IV-V (matured) gametocytes seem to exhibit a different AP2-G2-hPTM interaction. A middle-down proteomic dataset revealed that a unique triple hPTM activating complex, H3R17me2K18acK23ac, exclusively recruits AP2-G2 [76]. The resulting complex coordinates the formation of Spt-Ada-Gcn5 acetyltransferase like complex (SAGA like complex) through an interaction with a host of male-specific effector proteins, such as Chromodomain helicase DNA binding protein 1 (CHD1), General control non-repressed 5 protein (GCN5), Transcriptional adapter protein 2 (ADA2), Plant homodomain protein 2 (PHD2), Nucleosome assembly protein (NAPS), and Imitation Switch protein (ISWI) [76]. The interplay of AP2-G2 and the male-specific proteins alludes to the involvement of AP2-G2 in the regulation of male gametocytes as observed previously [72]. It is worthwhile to note that across eukaryotic cells, SAGA complexes are characterized as cellular coactivators that control transcription [77]. Against the interactions of AP2-G2 with H3R17me2K18acK23ac and the SAGA-like complex, it is however tempting to speculate that AP2-G2 is a transcriptional activator of sex-specific genes at the late gametocyte stages IV-V. This proposed activating function of AP2-G2 explains the reduced expression of essential late gametocyte markers in *ap2-g2*-disrupted phenotypes observed in previous studies [12, 48, 68]. This further strengthens the hypothesis that AP2-G2 is bifunctional and acts primarily as a repressor and also an activator of a subset of late gametocyte (stages IV-V) sex-specific genes. Nonetheless, further studies will be crucial to elucidate this hypothesis and also unravel the complexities that may be involved.

3.3. Regulation of Gametocytogenesis by Ap2-G3/FG

3.3.1. Phenotypes of AP2-G3/FG. Across available literature, the stage expression of AP2-G3 is marked by contradictions among different or same *Plasmodium* species. In *P. falciparum*, AP2-G3 appears not to be enriched in committed sexual rings or trophozoite [48, 78, 79]. However, Usui et al. [28] observed AP2-G3 enrichment in asexual rings. In addition, van Biljon et al. [48] observed a mild expression of AP2-G3 among stages II-IV gametocytes in *P. falciparum* (Figure 2). Conversely, to what is generally observed in *P. falciparum*, AP2-G3 seems to be substantially expressed among asexual parasites of *P. yoelii* and *P. berghei* before sexual commitment. In both *P. falciparum* and *P. yoelii*, it is predicted that AP2-G3 acts to transduce cytoplasmic signals into the nucleus which subsequently augments the transcription of *ap2-g* (Figure 3(b)) [28, 60].

AP2-G3, also described as AP2-FG in *P. berghei* by Yuda et al. [45], was putatively shown to activate the divergence of gametocytes into matured female gametocytes. Phenotypes of *ap2-g3*-disrupted parasites develop as immature female gametocytes, and this reflected a marked reduction of P47, NEK2, NEK3, P28, P25, putative ookinete proteins (PSOPs), CPW-WCP family proteins, and LAP family of proteins [45]. However, the development of *ap2-g3*-disrupted phenotypes into female gametocytes was not completely abrogated [45], which implies that AP2-G3 is not the molecular switch but rather a composite of a cascade of sex-specific gene

regulators. Notably, no significant effects on matured male gametocytes were reported in this study [45]. Parallel to this, in *P. yoelii*, disruption in *ap2-g3* substantially reduced the production of both matured male gametocytes and female gametocytes [60]. Thus, although there may be species differences, it seems that AP2-G3 has its regulatory mark on the commitment phase (trigger AP2-G expression) and sexual maturation/determination. Nonetheless, further studies are needed to unravel other functions of AP2-G3, owing to the scarcity of available studies.

3.3.2. Regulation of AP2-G3/FG. There is a paucity of data on the transcriptional regulation of AP2-G3. Using ChIP-seq, it was shown that AP2-G binding site was enriched upstream of AP2-G3 [11]. This suggests that AP2-G may directly regulate the expression of AP2-G3 at the transcription level.

Environmental cues such as high parasitaemia, anaemia, lysophosphatidylcholine (LysoPC), drug and immune pressure, and endoplasmic reticulum stress are associated with gametocyte commitment [80]. Against the model of AP2-G3/FG as a conduit for transmitting sexual commitment signals from the cytoplasm into the nucleus, AP2-G3 expression is likely influenced epigenetically by these environmental cues, which consequently impacts the expression of AP2-G. It is thought that LysoPC does not directly activate any molecular cascade, but rather, it causes a change within the intracellular space which consequently impacts epigenetic regulation of gametocytogenesis [81]. However, it remains to be elucidated how all known environmental cues of sexual commitment empirically influence AP2-G3 expression. Thus, a firm understanding of the potential mechanism(s) that underlines the activation of AP2-G3 by environmental cues seems lacking.

3.4. Regulation of Gametocytogenesis by AP2-G5

3.4.1. Phenotypes of AP2-G5. Recently, a comprehensive ChIP-seq analysis predicted the binding motif of *Pf*AP2-G5 as a five-based genomic sequence, GAACA or AACAA [66]. A CRISPR-Cas9 mediated disruption or deletion of *ap2-g5* in *P. falciparum* NF54 clones resulted in the upregulation of AP2-G suggesting that AP2-G5 is a repressor of *ap2-g* [66]. Comparative transcriptome analysis of a single *ap2-g* knockout and a dual *ap2-g5-ap2-g* knockout *P. falciparum* lines showed that AP2-G5 represses *ap2-g*, which consequently reduces the expression of essential AP2-G binding genes required for sexual commitment [66]. Thus, AP2-G5 prevents the initiation of sexual commitment. Further investigation showed that *Pf*AP2-G5 suppresses and prevents the untimely expression of gametocyte maturation-essential genes at the sexual ring stage. Thus, it was thought that repression of early gametocyte genes by AP2-G5 at the sexual ring stage promotes the maturation of early gametocytes. Nonetheless, there is a paucity of studies on AP2-G5, and as such, future investigations are needed to unravel its other roles.

3.4.2. Regulation of AP2-G5. An effort to delineate transcriptional regulation of AP2-G5 revealed that *ap2-g5* is activated by AP2-G to express AP2-G5 which in turn represses *ap2-g* consequently reducing the expression of early gametocyte genes. Thus, AP2-G recruits AP2-G5 as a regulatory molecule to keep in check its activities [66]. Currently, there is no concrete data on how the expression of AP2-G5 is epigenetically regulated.

3.5. Regulation of Gametocytogenesis by Other ApiAP2 Genes. In *P. yoelii*, AP2-O3 has been demonstrated to be exclusively expressed in matured female gametocytes but not in male gametocytes [44]. Further finding from Li et al. [44] showed that the disruption of *ap2-o3* in *P. yoelii* stalls the maturation of female gametocytes around stages II-III. The arrest of female gametocyte maturation in *ap2-o3* mutants is attributed to the reduced expression of female-specific genes (Figure 3(e)). Of notice, the downregulation of AP2-O3 in mutant *P. yoelii* parasite lines correlates with the upregulation of male-specific genes [20]. In addition, AP2-O in *P. falciparum* and AP2-SP/EXP in *P. berghei* are also implicated in gametocytogenesis (Table 1). For instance, phenotypes of AP2-O knockdown parasites have been shown to exhibit increased expression of many early gametocyte transcripts including *Pf*s16, suggesting that AP2-O perhaps is a repressor of these early gametocyte genes [82]. Also, in *P. berghei*, AP2-SP/EXP seems an indirect negative regulator of female-specific genes in gametocytes [75]. However, it remains to be elucidated how AP2-SP/EXP mediates the downregulation of female-specific genes. Further, in *P. falciparum*, stage I gametocytes are significantly enriched in AP2-SP2, AP2-O, and AP2-O3, but their functional relevance at this stage is unknown [48].

4. Conclusion

In the last decade, studies on functional genomes, transcriptomes, and proteomes have garnered impressive data on the transcriptional control of gametocytogenesis, and these have indeed illuminated in our understanding of a previously less-explored life stage of *Plasmodium* species. Keynotes from this review suggest that the role of AP2-G in sexual commitment and sexual conversion is conserved across different species and thus may present a more formidable target for transmission-blocking vaccines or drugs. Furthermore, the control of each step of gametocytogenesis by ApiAP2 genes is combinatorial and thus requires the effect of more than one ApiAP2 gene. For instance, the sexual commitment and sexual conversion stages are regulated primarily by AP2-G but do so alongside adjunct ApiAP2 players including AP2-G2, AP2-G3, and AP2-G5. The presence of the noncoding semiconserved intergenic region [83] within the ApiAP2 genome landscape likely provides avenues for the combinatorial control of a common phenotype by promoting the recruitment of other ApiAP2 genes. Nonetheless, there is very little homogeneity in the regulation of sexual maturation among different *Plasmodium* species, even by syntenic ortholog ApiAP2 genes. These observed discrepancies could be explained by (i) phenotypic plasticity that gives discrete genotypic clones the ability to express well-adapted phenotypes when exposed to different environments (different *in vivo* culturing environments); (ii) the

subtle difference in epigenetic regulation of gametocyte maturation among different *Plasmodium* species yielding varied phenotypes [84]; and (iii) the different gene-disruption techniques (knockout, knockdown, and genome editing) employed by the different studies which may or may not completely abolish the function of the target gene and hence produce different phenotypes.

It is important however to note that the hunt to unveil transcriptional roles of ApiAP2 genes, especially in gametocytogenesis, is still embryonic and has questions yet to be addressed. Importantly, a dataset obtained from a high-resolution transcriptome of *P. falciparum* has shown that, of 27 members of the ApiAP2family, 15 are substantially expressed during gametocytogenesis (Figure 2) [48], suggesting they might play important roles in gametocytogenesis. Nonetheless, only five of these genes have extensively characterized roles in gametocytogenesis but the rest are not known. Future investigations could focus on characterizing the function of these genes in gametocytogenesis and the pathways in which they participate for a holistic understanding of how ApiAP2 genes regulate gametocytogenesis. Strikingly, there is no or very little exploration of the functional control of gametocytogenesis by ApiAP2 genes in *P. vivax* and *P. malariae*, which are important *Plasmodium* species that pose a considerable health threat in South Asia, Central America, and South America [85]. Therefore, future studies could explore the roles of ApiAP2 genes in *P. vivax* and *P. malariae*.

Interestingly, using PacBio sequencing, it has been shown that there are many splicing isoforms of PF3F7_0730300, PF3F7_0420300, and PF3F7_0613800_1239200 ApiAP2 genes [86], although the roles of these isoforms are not yet functionally characterized. Alternative, splicing produces distinct mRNA isoforms from a single gene and thereby alters the structure and function of the resulting expressed proteins [87] and thus may hold cues to the observed different phenotypes produced by syntenic orthologs of ApiAP2 genes. Considering the crucial role of alternative splicing in the regulation of essential genes [87], future research trajectory could be directed toward the explorations of its roles in gametocytogenesis using other long-read sequencing techniques such as nanopore sequencing [88].

Data Availability

All data used to support the findings of this study are included within the article.

Disclosure

The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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