# *Plasmodium* sexual differentiation: how to make a female

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**Abstract**

Sexual development is integral to the transmission of *Plasmodium* parasites between vertebrates and mosquitos. Recent years have seen great advances in understanding the gene expression that underlies commitment of asexual parasites to differentiate into sexual gametocyte stages, then how they mature and form gametes once inside a mosquito. Less well understood is how parasites differentially control development to become males or females. *Plasmodium* parasites are haploid at the time of sexual differentiation, but a clonal haploid line can produce both male and female gametocytes, so they presumably lack the sex-determining alleles present in some other eukaryotes. Though the molecular switch to initiate male or female development remains hidden, recent studies reveal regulatory proteins needed for the sex-specific maturation of male and female gametocytes. In this issue, Yuda and collaborators report the characterization of a transcription factor necessary for female gametocyte maturation. With renewed attention on malaria elimination, sex has been an increasing focus as transmission-blocking strategies are likely to be an important component of elimination efforts.

**Introduction**

To transmit from vertebrate to mosquito hosts, *Plasmodium* parasites must form sexually-differentiated gametocytes that can be ingested in a mosquito blood meal. Inside the mosquito midgut, these haploid gametocytes quickly transform into male and female gametes, which must fuse to form diploid zygotes in order to continue the life cycle. Parasites within a vertebrate must strike a balance between forming replicating asexual stage parasites that maintain an infection and producing enough non-proliferative gametocyte to ensure transmission (Schneider *et al.*, 2018). The rate of commitment to gametocyte forms compared to asexual linages varies between parasite lines and between environmental conditions, suggesting that an interplay between host and pathogen factors influences gametocyte commitment (Josling *et al.*, 2018).

A transcription factor of the ApiAP2 family termed PfAP2-G is the conserved master regulator of sexual conversion in all *Plasmodium* species (Kafsack *et al.*, 2014, Sinha *et al.*, 2014, Zhang *et al.*, 2017). The current model stands that in asexual parasites, the *ap2-g* gene is silenced by epigenetic mechanisms that involve heterochromatin at this locus. Activation of the gene, which in *P. falciparum* requires displacement of the heterochromatin protein 1 (HP1) in a process that depends on the gametocyte development 1 protein (GDV1) (Filarsky

*et al.*, 2018) , results in sexual commitment. Several downstream regulators, including the transcriptional repressor AP2-G2 (Sinha *et al.*, 2014, Yuda *et al.*, 2015), contribute to the following steps of sexual differentiation. In addition to the "choice" between asexual and gametocyte lineages, there is another other step where alternative developmental options are possible for the parasite: to become either male or female gametocyte. As with the basic commitment to sexual stages, the differential production and survival of males and females relies on parasite and host factors (Tadesse *et al.*, 2019). However, why, how, and when parasites become male or female is incompletely understood.

Working with the murine malaria parasite *P. berghei*, Yuda and colleagues shed some light to the mechanism of female gametocyte development with the description of an ApiAP2 transcription factor previously named AP2-G3 and here referred to as AP2-FG (FG stands for female gametocyte) (Yuda *et al.*, 2019). They show that this transcription factor is expressed specifically in female gametocytes. Disruption of the gene results in arrested maturation of female gametocytes at an early stage of development, which makes them non-viable for productive mosquito infection. In contrast, the development of male gametocytes is not affected by absence of this protein. However, early female gametocytes are observed in parasite lines lacking AP2-FG, indicating that other factors operating upstream determine the male or female sex. These data suggests that AP2-FG plays a specific role in driving the expression of genes necessary for female gametocyte maturation. In support of this view, disruption of AP2-FG results in reduced expression of a number of female-transcribed genes (Yeoh *et al.*, 2017), and Chip-Seq analyses show that this protein binds preferentially to the regulatory region of genes involved in various processes in female gametocyte biology. Binding appears to occur via a newly identified 10 bp motif (Yuda *et al.*, 2019). Altogether, Yuda et al. provide compelling evidence for the first clear identification of a transcription factor that regulates sex-specific expression.

**Differences between species**

Intriguingly, a previous study in *P. berghei* was unable to generate a knockout of AP2-FG in asexual stages, suggesting a potential role in this phase (Modrzynska *et al.*, 2017), whereas disruption of the orthologous gene in *P. yoelii* (where it was named AP2-G3) produced a more profound reduction in male than in female gametocytes (Zhang *et al.*, 2017). In *P. yoelii*, AP2-FG/AP2-G3 was proposed to play a role in gametocyte development upstream of AP2-G. The data regarding the stage-specificity of the expression of this gene are also

conflicting. Previous transcriptomic studies didn't detect an enrichment in *P. falciparum* gametocytes or asexual parasites commited to gametocytogenesis (Le Roch *et al.*, 2003, Pelle *et al.*, 2015, Lopez-Barragan *et al.*, 2011), or reported only a mild increase in transcript abundance in females compared to males (Lasonder *et al.*, 2016). Likewise, RNAseq experiments in *P. berghei* also show no enrichment of AP2-FG/AP2-G3 in gametocytes (Otto *et al.*, 2014) , and transcript abundance is higher in males rather than in females (Yeoh *et al.*, 2017).

There are several possible explanations for the apparent discrepancies. First, transcript levels of this transcription factor may increase in the female lineage only for a very short time as an intermediate step in a cascade of transcription factors. Transcriptional analysis of bulk purified male or female gametocytes includes mRNA from a wide temporal window, so might miss transient expression early in the female lineage. Second, post-transcriptional control mechanisms are known to play a major role during sexual development (Mair *et al.*, 2006, Shrestha *et al.*, 2016, Miao *et al.*, 2010). Differential post-transcriptional regulation of AP2-FG between parasites at different stages or of different sex may explain the discrepancy between the female-specific expression of AP2-FG-GFP fusion proteins reported by Yuda et al. and the more promiscuous expression of *ap2-fg* transcripts described by others. Last, while the regulation of some steps of sexual development appears to be conserved among malaria species (e.g. commitment mediated by activation of the master regulator *ap2-g*), other steps may rely on different regulators in different *Plasmodium* species. For example, the above-mentioned GDV1 is present in human-infecting *Plasmodium* species but absent in many other species. In this regard, it is important to mention that gametocyte maturation differs dramatically between species in its duration and in the morphological changes that ensue (Ngotho *et al.*, 2019). Caution in extrapolating the function of ApiAP2 proteins from one species to another is warranted.

**How and when do parasites undergo sex determination?**

Until recently, the prevailing model was that once a parasite commits to sexual development, it must go through an additional round of replication before starting to differentiate, such that all merozoites arising from the same schizont produce only asexual forms or only gametocytes (Bruce *et al.*, 1990). However, recent research in *P. berghei* and *P. falciparum* has shown that parasites can also commit to sexual development and start differentiating into sexual forms within the same cycle (Kent *et al.*, 2018, Bancells *et al.*, 2019) . It is generally

accepted that the commitment to become a male or female is tightly linked with the overall commitment to become a gametocyte, or occurs soon thereafter. Evidence for this model is based on plaque assays, where schizonts are allowed to develop in a monolayer of immobilized erythrocytes. In such assays, plaques of parasites arising from the same schizont generally contain only male gametocytes or only female gametocytes, rather than a mixture of both (Smith *et al.*, 2000, Silvestrini *et al.*, 2000).

Nonetheless, several non-mutually exclusive routes to sexual differentiation are possible. **In scenario 1** (Figure 1), parasites receive a transcriptional signal mediated by AP2-G production, that drives them to differentiate into gametocytes (Sinha *et al.*, 2014, Kafsack *et al.*, 2014). These parasites simultaneously commit to becoming either male or female, driven by as-yet-undiscovered male- or female-specific transcription factors. Parasites undergo an additional round of replication, but offspring of the committed progenitor will be all male sexual gametocytes, or all female sexual gametocytes, and not a mixture of both. **Under scenario 2**, ring stage parasites receive a transcriptional signal driven by AP2G to differentiate into gametocytes without undergoing a further proliferative cycle, and simultaneously commit to becoming either male or female, driven by sex-specific transcription factors. **Under scenario 3**, parasites first receive a transcriptional signal to differentiate into gametocytes, with or without and additional round of multiplication, but only later during sexual development commit to becoming either male or female. This scenario would predict that parasites arising from the same schizont could form a mixture of male and female gametocytes, which has not been observed so far in plaque assays. Additional scenarios may involve a "default sex" that will develop and mature in the absence of diversion to the other sex, as in some other sexual organisms. It is also formally possible that no transcription factors are involved and sexual dimorphism is regulated only at other levels (e.g. post-transcriptionally).

**Initiation versus maturation of sexual forms**

Whatever the route to male-female differentiation, the observation that parasites that lack AP2-FG still initiate female commitment and start expressing female-specific markers (Yuda *et al.*, 2019) indicates that AP2-FG is not itself the switch that determines female sex, but rather part of a regulatory cascade that is initiated by other factors that precede AP2-FG. So far, AP2-G is the only known ApiAP2 transcriptional regulator that operates as a developmental switch, whereas other members of the ApiAP2 family, including AP2-FG,

regulate the expression of specific genes as part of a regulatory cascade.

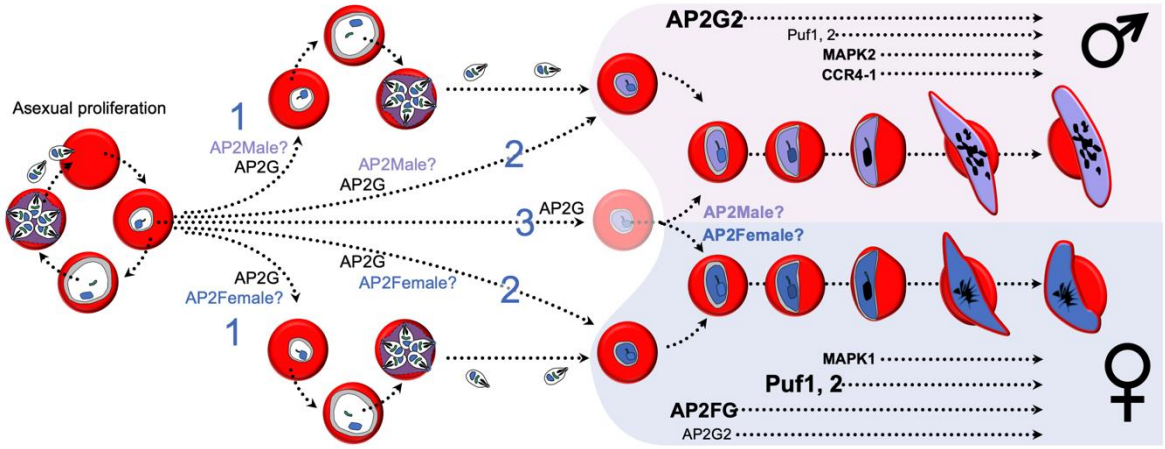
AP2-FG is unique in that in *P. berghei* it plays a highly specific role in female gametocytes, but several other factors also show some level of sex-specificity in their function (Figure 1): disruption of the transcriptional repressor AP2-G2 is more detrimental to males than females (Sinha *et al.*, 2014), and the disruption of the translational repressors Puf1 and Puf2 preferentially inhibits the development of female gametocytes (Shrestha *et al.*, 2016, Miao *et al.*, 2010). The mitogen-activated protein kinases MAPK1 and MAPK2 are additional candidate regulators of female and male-specific maturation, respectively (Walzer *et al.*, 2018). Another translational repressor, CCR4-1, is required for normal development of male gametes in *P. yoelii* (Hart *et al.*, 2019). As well as these post-transcriptional actors, a large number of uncharacterised ApiAP2 transcription factors have been identified as being differentially expressed between males and females (Yeoh *et al.*, 2017), and timecourses of gametocyte development (Kent *et al.*, 2018) provide temporal data on their order of expression. These ApiAP2s are prime candidates for maturation factors for each sex, as well as potential master-switches for male or female commitment.

**Concluding remarks**

In recent years there has been impressive progress in our understanding of the regulation of life cycle progression in malaria parasites. In all cases studied so far, developmental transitions involve ApiAP2 DNA binding proteins (Jeninga *et al.*, 2019). The work from Yuda et al. provides the first identification of a female-specific transcription factor. Notably, the characterization of the ApiAP2s that regulate specific transitions has revealed a level of complexity that rules out a simple model in which a linear cascade of transcription factors operates with each ApiAP2 regulating non-overlapping sets of genes. Instead, a more intricate model is emerging in which cooperative interactions or competition between ApiAP2s dominate, and some factors have functions at multiple stages. This enables the regulation of a complex life cycle with fewer than 30 ApiAP2 transcription factors, in concert with epigenetic factors (van Noort & Huynen, 2006, Josling *et al.*, 2019, Jeninga *et al.*, 2019). The data from Yuda et al. supports this view, as the majority of AP2-FG targets (as determined by ChIP-seq) are still expressed in the KO parasite lines, albeit at lower levels. This suggests that other transcription factors and epigenetic regulators contribute to the expression of these genes. Future studies are expected to unravel the full complexity of the ApiAP2 regulatory network in malaria parasites.

**Figure legend**

**Fig. 1. Hypothetical routes to sexual differentiation.** Under scenarios **1** and **2**, sexual differentiation is determined by male- and female-specific transcription factors (depicted here as the hypothetical AP2-Male and AP2-Female), activated concomitantly with PfAP2-G, with (**scenario 1**) or without (**scenario 2**) an additional cycle of replication after commitment (marked by PfAP2-G expression). Under **scenario 3**, parasites start developing as sexual forms and only later initiate dimorphic sexual differentiation once sex-specific factors are activated. Factors involved in male or female development, including AP2-FG, are indicated. Font size reflects the relative importance for male and female development.




**References**


Bancells, C., Llora-Batlle, O., Poran, A., Notzel, C., Rovira-Graells, N., Elemento, O., Kafsack, B.F.C., and Cortes, A. (2019) Revisiting the initial steps of sexual development in the malaria parasite *Plasmodium falciparum*. *Nat Microbiol* **4**: 144-154.

Bruce, M.C., Alano, P., Duthie, S., and Carter, R. (1990) Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* **100 Pt 2**: 191-200.

Filarsky, M., Fraschka, S.A., Niederwieser, I., Brancucci, N.M.B., Carrington, E., Carrio, E., Moes, S., Jenoe, P., Bartfai, R., and Voss, T.S. (2018) GDV1 induces sexual

commitment of malaria parasites by antagonizing HP1-dependent gene silencing. *Science* **359**: 1259-1263.

Hart, K.J., Oberstaller, J., Walker, M.P., Minns, A.M., Kennedy, M.F., Padykula, I., Adams, J.H., and Lindner, S.E. (2019) *Plasmodium* male gametocyte development and transmission are critically regulated by the two putative deadenylases of the CAF1/CCR4/NOT complex. *PLoS Pathog* **15**: e1007164.

Jeninga, M.D., Quinn, J.E., and Petter, M. (2019) ApiAP2 Transcription Factors in Apicomplexan Parasites. *Pathogens* **8**.

Josling, G.A., Venezia, J., Orchard, L., Russell, T.J., Painter, H.J., and Llinas, M. (2019) Regulation of sexual differentiation is linked to invasion in malaria parasites. *bioRxiv*: 533877.

Josling, G.A., Williamson, K.C., and Llinas, M. (2018) Regulation of Sexual Commitment and Gametocytogenesis in Malaria Parasites. *Annu Rev Microbiol* **72**: 501-519.

Kafsack, B.F., Rovira-Graells, N., Clark, T.G., Bancells, C., Crowley, V.M., Campino, S.G., Williams, A.E., Drought, L.G., Kwiatkowski, D.P., Baker, D.A., Cortes, A., and Llinas, M. (2014) A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature* **507**: 248-252.

Kent, R.S., Modrzynska, K.K., Cameron, R., Philip, N., Billker, O., and Waters, A.P. (2018) Inducible developmental reprogramming redefines commitment to sexual development in the malaria parasite *Plasmodium berghei*. *Nat Microbiol* **3**: 1206-1213.

Lasonder, E., Rijpma, S.R., van Schaijk, B.C., Hoeijmakers, W.A., Kensche, P.R., Gresnigt, M.S., Italiaander, A., Vos, M.W., Woestenenk, R., Bousema, T., Mair, G.R., Khan, S.M., Janse, C.J., Bartfai, R., and Sauerwein, R.W. (2016) Integrated transcriptomic and proteomic analyses of *P. falciparum* gametocytes: molecular insight into sex-specific processes and translational repression. *Nucleic Acids Res* **44**: 6087-6101.

Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., De La Vega, P., Holder, A.A., Batalov, S., Carucci, D.J., and Winzeler, E.A. (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301**: 1503-1508.

Lopez-Barragan, M.J., Lemieux, J., Quinones, M., Williamson, K.C., Molina-Cruz, A., Cui, K., Barillas-Mury, C., Zhao, K., and Su, X.Z. (2011) Directional gene expression and antisense transcripts in sexual and asexual stages of *Plasmodium falciparum*. *BMC Genomics* **12**: 587.

Mair, G.R., Braks, J.A., Garver, L.S., Wiegant, J.C., Hall, N., Dirks, R.W., Khan, S.M., Dimopoulos, G., Janse, C.J., and Waters, A.P. (2006) Regulation of sexual development of Plasmodium by translational repression. *Science* **313**: 667-669.

Miao, J., Li, J., Fan, Q., Li, X., Li, X., and Cui, L. (2010) The Puf-family RNA-binding protein PfPuf2 regulates sexual development and sex differentiation in the malaria parasite *Plasmodium falciparum*. *J Cell Sci* **123**: 1039-1049.

Modrzynska, K., Pfander, C., Chappell, L., Yu, L., Suarez, C., Dundas, K., Gomes, A.R., Goulding, D., Rayner, J.C., Choudhary, J., and Billker, O. (2017) A Knockout Screen of ApiAP2 Genes Reveals Networks of Interacting Transcriptional Regulators Controlling the Plasmodium Life Cycle. *Cell Host Microbe* **21**: 11-22.

Ngotho, P., Soares, A.B., Hentzschel, F., Achcar, F., Bertuccini, L., and Marti, M. (2019) Revisiting gametocyte biology in malaria parasites. *FEMS Microbiol Rev*.

Otto, T.D., Bohme, U., Jackson, A.P., Hunt, M., Franke-Fayard, B., Hoeijmakers, W.A., Religa, A.A., Robertson, L., Sanders, M., Ogun, S.A., Cunningham, D., Erhart, A., Billker, O., Khan, S.M., Stunnenberg, H.G., Langhorne, J., Holder, A.A., Waters, A.P., Newbold, C.I., Pain, A., Berriman, M., and Janse, C.J. (2014) A comprehensive evaluation of rodent malaria parasite genomes and gene expression. *BMC Biol* **12**: 86.

Pelle, K.G., Oh, K., Buchholz, K., Narasimhan, V., Joice, R., Milner, D.A., Brancucci, N.M., Ma, S., Voss, T.S., Ketman, K., Seydel, K.B., Taylor, T.E., Barteneva, N.S., Huttenhower, C., and Marti, M. (2015) Transcriptional profiling defines dynamics of parasite tissue sequestration during malaria infection. *Genome Med* **7**: 19.

Schneider, P., Greischar, M.A., Birget, P.L.G., Repton, C., Mideo, N., and Reece, S.E. (2018) Adaptive plasticity in the gametocyte conversion rate of malaria parasites. *PLoS Pathog* **14**: e1007371.

Shrestha, S., Li, X., Ning, G., Miao, J., and Cui, L. (2016) The RNA-binding protein Puf1 functions in the maintenance of gametocytes in *Plasmodium falciparum*. *J Cell Sci* **129**: 3144-3152.

Silvestrini, F., Alano, P., and Williams, J.L. (2000) Commitment to the production of male and female gametocytes in the human malaria parasite *Plasmodium falciparum*. *Parasitology* **121 Pt 5**: 465-471.

Sinha, A., Hughes, K.R., Modrzynska, K.K., Otto, T.D., Pfander, C., Dickens, N.J., Religa, A.A., Bushell, E., Graham, A.L., Cameron, R., Kafsack, B.F.C., Williams, A.E., Llinas, M., Berriman, M., Billker, O., and Waters, A.P. (2014) A cascade of DNA-binding

proteins for sexual commitment and development in *Plasmodium*. *Nature* **507**: 253-257.

Smith, T.G., Lourenco, P., Carter, R., Walliker, D., and Ranford-Cartwright, L.C. (2000) Commitment to sexual differentiation in the human malaria parasite, *Plasmodium falciparum*. *Parasitology* **121 ( Pt 2)**: 127-133.

Tadesse, F.G., Meerstein-Kessel, L., Goncalves, B.P., Drakeley, C., Ranford-Cartwright, L., and Bousema, T. (2019) Gametocyte Sex Ratio: The Key to Understanding *Plasmodium* falciparum Transmission? *Trends Parasitol* **35**: 226-238.

van Noort, V., and Huynen, M.A. (2006) Combinatorial gene regulation in *Plasmodium falciparum*. *Trends Genet* **22**: 73-78.

Walzer, K.A., Kubicki, D.M., Tang, X., and Chi, J.T. (2018) Single-Cell Analysis Reveals Distinct Gene Expression and Heterogeneity in Male and Female *Plasmodium falciparum* Gametocytes. *mSphere* **3**.

Yeoh, L.M., Goodman, C.D., Mollard, V., McFadden, G.I., and Ralph, S.A. (2017) Comparative transcriptomics of female and male gametocytes in *Plasmodium berghei* and the evolution of sex in alveolates. *BMC Genomics* **18**: 734.

Yuda, M., Iwanaga, S., Kaneko, I., and Kato, T. (2015) Global transcriptional repression: An initial and essential step for *Plasmodium* sexual development. *Proc Natl Acad Sci U S A* **112**: 12824-12829.

Yuda, M., Kaneko, I., Iwanaga, S., Murata, Y., and Kato, T. (2019) Female-specific gene regulation in malaria parasites by an AP2-family transcription factor. *Molecular Microbiology* **In this issue - INCOMPLETE**.

Zhang, C., Li, Z., Cui, H., Jiang, Y., Yang, Z., Wang, X., Gao, H., Liu, C., Zhang, S., Su, X.Z., and Yuan, J. (2017) Systematic CRISPR-Cas9-Mediated Modifications of *Plasmodium yoelii* ApiAP2 Genes Reveal Functional Insights into Parasite Development. *MBio* **8**.