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Sex: How Malaria Parasites Get Turned On

The mechanisms underlying sexual stage switching in *Plasmodium* spp. have hitherto remained a mystery. However, two recent studies have revealed that an apicomplexan-specific DNA-binding protein is essential for the initiation of this cell fate decision, ultimately providing the malaria community with a novel and important tool in the battle to prevent malaria transmission.

Johan Ankarklev, Nicolas M.B. Brancucci, Ilana Goldowitz, Pierre-Yves Mantel, and Matthias Marti*

Sex is one of the central innovations of the eukaryote domain and a key to its vast success. As such, the germ lines are ultimately responsible for the evolution of a sexually reproducing species; the information contained in the germ line determines what will be passed on from one generation to the next. Eukaryotes use widely varied mechanisms for sexual reproduction but a common theme is the initiation or segregation of germ cells from the somatic or asexual population. In yeast, induction of mating types and sporulation commonly occur in response to pheromones or environmental factors [1]. Among the metazoans, germ cell specification transpires through two major pathways: epigenesis (induction through external signals) or preformation (localization of maternally inherited determinants) [2].

Sex in the protozoa can be difficult to observe and in many species occurs in unusual ways or has never been described. Nevertheless, sex is part of the life cycle in all members of the exclusively parasitic apicomplexan phylum [3]. Many of these unicellular species undergo 'traditional' meiosis; among them are the *Plasmodium* spp., the causative agents of malaria [4]. Apicomplexan organisms engage in complex life cycles in which only one stage is capable of sexual reproduction, analogous to the alternation of generations seen in fungi, plants, and eukaryotic algae. In *Plasmodium*, seven stages develop sequentially as the parasite moves through different microenvironments in its vertebrate host and its mosquito vector. The progression from one stage to the next is linear and predetermined in all cases but one: the switch from the asexually replicating form in vertebrate red blood cells to the sexual stage capable of transmitting to the mosquito vector, initiated by a differentiation step. Some apicomplexan species such as *Hepatozoon* are believed to always proceed directly to sexual reproduction in a predetermined manner, whereas in *Plasmodium* spp. each asexual schizont stage is hypothesized to choose between asexual and sexual replication through a combination of environmental factors present in the host microenvironment in parallel with innate genetic factors (Figure 1) [3]. Environmental factors proposed to aid in this decision include host immunity, host hormones and certain anti-malarial drugs [5]. More recently, infected red blood cell derived (iRBC) extracellular vesicles (EVs) were shown to stimulate the switch to the sexual stage, also known as gametocytogenesis, in a density-dependent manner [6]. The genetic factors underlying the

induction of sexual differentiation in *Plasmodium* and other Apicomplexa have until now remained elusive. Because gametocytes are the stage responsible for transmission from humans to mosquitoes, this lack of information has hampered efforts to control malaria. Substantial effort has been put into studying the vertebrate asexual cycle, but in order to prevent transmission, obstruction of sexual stage development is imperative.

Two recent papers by Kafsack *et al.* and Sinha *et al.* reveal that an apicomplexan-specific transcription factor, ApiAP2-G, is essential for sexual differentiation, both in the human pathogen *Plasmodium falciparum* [7] and in its murine relative *P. berghei* [8]. The apicomplexan AP2 (ApiAP2) family of DNA-binding proteins is homologous to the Apetela2/ethylene response factor (AP2/ERF) family found in plants [9]. ApiAP2 orthologs have been found in all apicomplexan genomes analyzed to date, including *Toxoplasma gondii*, *Theileria* spp. and *Cryptosporidium* spp. Recent insights in *Plasmodium* have implicated ApiAP2-mediated gene regulation in developmental processes across the intricate parasite life cycle [10]. These efforts have revealed that specific *Plasmodium* ApiAP2 genes are involved at different phases of gametocyte maturation, in the control of mosquito stage ookinete and oocyst development, and in regulation of gene expression in vertebrate liver stage parasites [11].

Kafsack *et al.* alongside Sinha and colleagues both set out to identify genetic factors that differ between gametocyte-deficient and gametocyte-producing *Plasmodium* cell lines. *Plasmodium* spp. parasites maintained either *in vitro* or by repeated passage through vertebrate hosts commonly lose their ability to produce sexual stage parasites over time [12]. Both teams discovered that strains with loss of gametocyte production had only one mutation in common, located in ApiAP2-G (*PFL1085w*) in *P. falciparum* and its ortholog *PBANKA_143750* in *P. berghei*. The teams verified that no loss-of-function mutations in ApiAP2-G exist in approximately 300 *P. falciparum* field strains. Furthermore, Kafsack and colleagues managed to elegantly link levels of ApiAP2-G transcripts to levels of gametocyte production among several

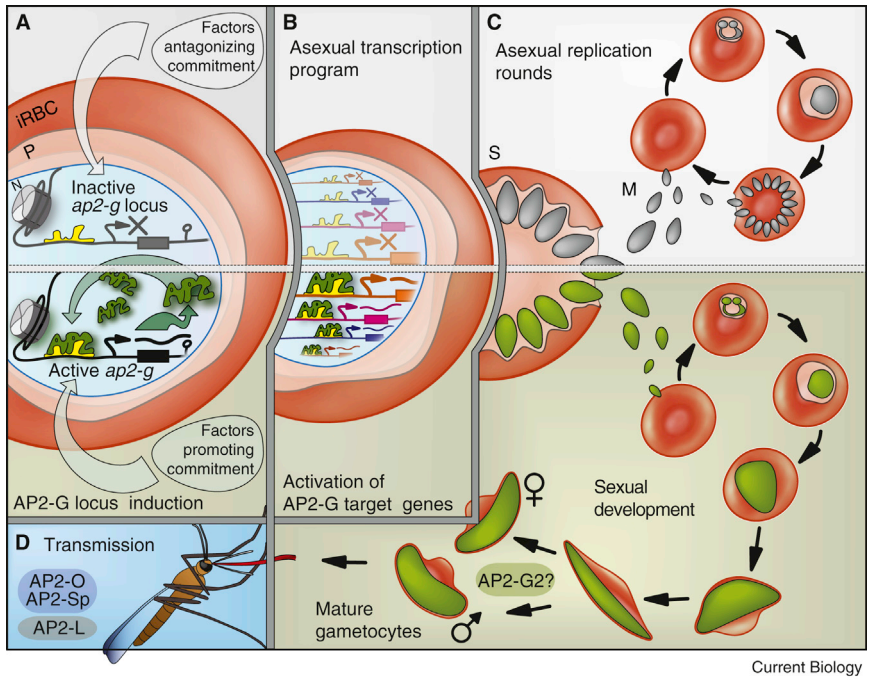

Figure 1. Model of AP2-G-induced sexual differentiation in *P. falciparum*.

(A) The *ap2-g* locus is depicted in either the transcriptionally inert (upper panels) or active state (lower panels). Once expressed, the AP2-G protein (shown in green) may enhance activity of its own coding region through specific binding to an upstream DNA motifs (in yellow). Various factors deriving from the environment, from the host and/or from the parasites themselves are suggested to modulate the rate of sexual differentiation. For example, the parasite may actively use micro-vesicle-mediated communication to modulate this cell fate decision in a density-dependent manner. The authors of the two recent studies suggest that the *ap2-g* locus is epigenetically controlled, and it will be necessary to dissect the underlying mechanisms in future studies. (B) AP2-G controls the activity of numerous target genes that, in turn, are required for the early sexual pathway. When the protein is absent, parasites fail to induce a gametocyte-specific gene cascade. (C) Non-committed merozoites (in gray) continue asexual reproduction after schizogony and erythrocyte re-invasion. In contrast, committed progeny (in green) enter sexual development. AP2-G2 may at some stage contribute to determining the sex ratio between transmissible gametocytes. (D) Following the mosquito blood meal, zygotes are formed and continue parasite development within the insect vector. Two members of the AP2 family, AP2-O and AP2-Sp, were previously shown to be essential for oocyst and sporozoite mosquito stage formation, respectively. After transmission to the mammalian host, another AP2 factor, AP2-L, is required for liver stage development, emphasizing the important role of this family of DNA-binding proteins in the developmental cycle of *Plasmodium* spp., and potentially of other Apicomplexa. (iRBC, infected red blood cell; P, parasite; N, parasite nucleus, S, schizont; M, merozoites.)

parasite lines [7]. Assays measuring direct competition between wild-type and mutant strains showed that mutations in AP2-G confer a growth advantage, likely explaining the common occurrence of gametocyte deficiency in long-term laboratory cultures. Kafsack *et al.* and Sinha *et al.* verified the essentiality of AP2-G for sexual stage production by conditional knock-down or targeted gene disruption, respectively, and by correcting mutations in the gene through complementation of the mutant strain. In subsequent *in vitro* or mouse infection assays, gametocytes were produced exclusively by strains with the respective wild-type *pfap2-g* or *pbap2-g* genes. Kafsack *et al.* went on to compare the expression levels of gametocyte-specific genes between a high gametocyte-producing line and two *pfap2-g* mutant lines over a 48 hour intra-erythrocytic cycle. The mutant clones displayed lower relative abundance of the earliest known gametocyte development genes in addition to expression changes in several other genes, demonstrating the protein's importance to very early gametocytogenesis events.

Sinha and colleagues went a step further and verified the involvement of a second member of the ApiAP2 family acting downstream of *PbAP2-G* in early gametocyte development, which they named *PbAP2-G2*. Disruption of this gene dramatically decreased the

number of gametocytes produced. Interestingly, the resulting population was completely devoid of male gametocytes but contained a small number of females, which suggests that *PbAP2-G2* is also a regulatory factor in sex determination. Although further studies are necessary to verify these findings, they are the first results highlighting a putative sex determination factor in malaria. The same authors utilized protein-binding arrays to assay target sites of the recombinant DNA-binding domain of *PbAp2-G* followed by electrophoretic mobility shift assays, which yielded the discovery of two palindromic hexameric motifs (GxGTAC and GTACxC) necessary for binding of this transcription factor. Notably, these motifs occur at a significantly higher frequency within the 2 kb upstream region of genes with known gametocyte-specific expression patterns, compared to non-gametocyte genes. Interestingly, both motifs were present upstream of *PbAP2-G* itself, which led the authors to suggest the potential of an autoregulatory positive feedback mechanism. Similar feedback events are known to occur in other unicellular organisms such as *Candida* spp., in which multiple transcription factors regulate sexual stage switching through a network of positive and negative feedback loops [13]. Furthermore, high levels of H3K9me3 histone modifications and the perinuclear localization of *pfap2-g*, along with the previously measured presence of heterochromatin protein HP1 [14], led Kafsack and colleagues to suggest that this gene is epigenetically silenced in the asexual cycle in a similar fashion as that seen among multigene families involved in virulence and immune evasion in *P. falciparum*. The *pfap2-g* locus could thus be epigenetically silenced during asexual replication but prone to activation, which in turn would explain the relatively low number of gametocytes seen in a given *Plasmodium* population. This idea deserves further investigation.

The studies performed by the authors described herein are the first to map and characterize specific factors involved in sexual stage commitment in *Plasmodium* spp. or in any other apicomplexan. Conceptually, AP2-G will be a powerful tool for the malaria community as it provides a genetic on/off switch for gametocytogenesis [7] and thus capacitates controlled experimental commitment assays. There are likely several other factors involved in the onset of gametocytogenesis, and future initiatives to map entire signaling and gene regulation pathways, both prior to and after the point at which ApiAP2-G acts, are now possible. Finally, the conserved nature of the ApiAP2 family among the Apicomplexa suggests that orthologs of ApiAP2-G or other AP2 transcription factors could contribute to sexual stage switching across this phylum. The lack of ApiAP2 orthologs in humans and other vertebrate hosts highlights ApiAP2-G as a strong candidate for the development of novel drugs targeting transmission of malaria in particular but potentially also of other apicomplexan pathogens.

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Cell Biology: ESCRTing Trouble Out!

Calcium entry through a plasma membrane defect leads to the local recruitment of endosomal complex required for transport (ESCRT) proteins. These proteins are hypothesized to drive an outward bending of the affected plasma membrane, forming a small bud that is then shed from the cell, along with the troublesome defect.

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Plasma membrane defects are created *in vivo* under physiological conditions that generate mechanical stress. The most well-studied example is skeletal muscle undergoing eccentric contraction exercise: this generates maximal levels of mechanical stress on muscle fibers and a dramatic rise in the incidence of plasma membrane disruption [1]. Pathological conditions can also produce disruptions, including of course traumatic injury and electrical shock. Moreover, bacteria liberate pore-forming toxins — proteins that lodge in the host cell plasma membrane, where, as multimeric transmembrane arrays, they form channels for abnormal