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Division and Transmission: Malaria Parasite Development in the Mosquito

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Abstract

The malaria parasite life cycle alternates between two hosts: a vertebrate and the female *Anopheles* mosquito vector. Cell division, proliferation, and invasion are essential for parasite development, transmission, and survival. Most research has focused on *Plasmodium* development in the vertebrate, which causes disease; however, knowledge of malaria parasite development in the mosquito (the sexual and transmission stages) is now rapidly accumulating, gathered largely through investigation of the rodent malaria model, with *Plasmodium berghei*. In this review, we discuss the seminal genome-wide screens that have uncovered key regulators of cell proliferation, invasion, and transmission during *Plasmodium* sexual development. Our focus is on the roles of transcription factors, reversible protein phosphorylation, and molecular motors. We also emphasize the still-unanswered important questions around key pathways in cell division during the vector transmission stages and how they may be targeted in future studies.

Contents

1. INTRODUCTION: DEVELOPMENT OF THE MALARIA PARASITE IN THE MOSQUITO

Malaria is a mosquito-borne disease caused by transmission of the apicomplexan parasite *Plasmodium*. The parasite is haploid during most of its life cycle, and the disease in the vertebrate host is manifested in the red blood cell asexual stage. During this replicative phase, some parasites commit to differentiating into sexual cells, male and female gametocytes that are arrested at the G0 phase of the cell cycle. Once ingested by a mosquito in a blood meal, the male (micro)gametocyte is activated and undergoes three rapid rounds of DNA replication and mitosis to produce eight haploid microgametes, which subsequently fertilize activated female (macro)gametes (**Figure 1**). The resultant diploid zygote undergoes the first stage of meiosis: a single round of DNA replication to produce a tetraploid cell, during differentiation and elongation into a mature ookinete, which then invades the mosquito midgut wall and develops into an oocyst. Here, multiple rounds of endomitotic division followed by cytokinesis produce hundreds of haploid sporozoites (a process termed sporogony) (31, 35), which are released from the oocyst and migrate to the salivary glands for transmission to the vertebrate host during the mosquito's next blood meal (**Figure 1**).

Sporogony: a complex process involving repeated cycles of genome replication without nuclear division, ultimately resulting in hundreds of haploid sporozoites

Different proliferative stages exhibit two unusual types of mitosis and a single phase of meiosis. The first type of mitosis occurs during preerythrocytic schizogony in liver hepatocytes, blood stage schizogony within host erythrocytes, and sporogony in the mosquito (5). In this asynchronous, closed endomitotic division that precedes cytokinesis, genome duplication and chromosome segregation are accomplished via a spindle within an intact nuclear membrane, and without some of the classical morphological features of mitosis, such as chromosome condensation (2, 35). The second type of atypical mitotic division is that within male gametocytes, where the three rounds

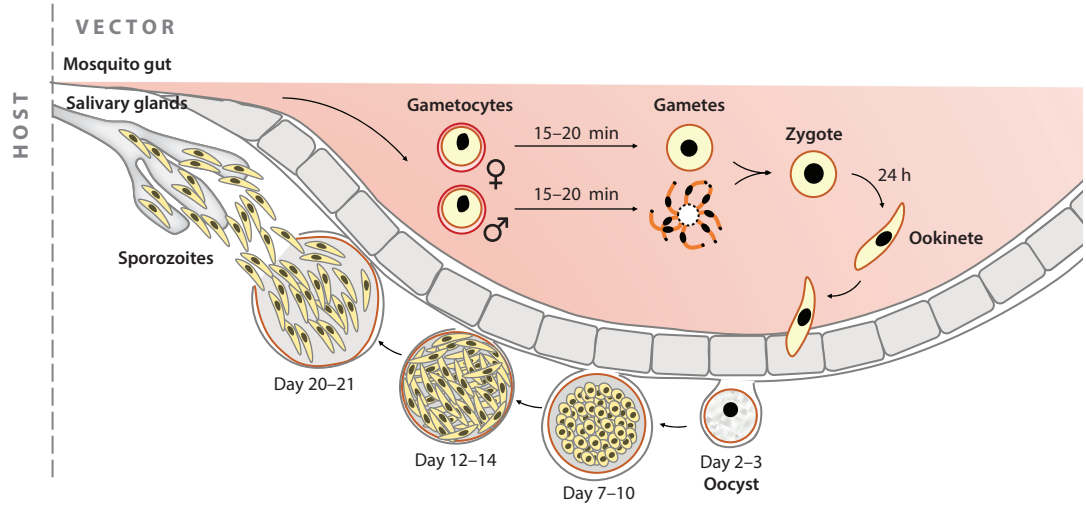

Figure 1

The *Plasmodium* life cycle inside the mosquito starts with ingestion of gametocytes (male and female sexual cells), which circulate in the host's bloodstream. These gametocytes further develop into gametes inside the mosquito gut. A zygote is formed after fertilization and transforms into a crescent-shaped ookinete with intermediate retort forms, where meiotic division occurs. Ookinetes migrate to and traverse the inner gut wall, forming oocysts on the outer surface of the gut. The oocysts further develop and produce hundreds of sporozoites after multiple rounds of endomitotic division. Eventually, the oocyst bursts and releases sporozoites that migrate to the salivary glands and are injected by the mosquito into a new host, thus beginning a new infection cycle.

of endo-reduplication (resulting in an octoploid nucleus) are followed by chromosome condensation and nuclear budding to produce haploid gametes (42, 55). The meiotic process is poorly understood; the tetraploid ookinete has four nuclear poles but no chromosome condensation (112, 144).

In this review, we discuss the regulatory processes controlling malaria parasite development and proliferation within the mosquito midgut, focusing on the molecular and functional genome-wide screens in *Plasmodium berghei* that revealed key players regulating transcription; reversible protein phosphorylation; and the molecular motors that drive cell division, proliferation, differentiation, motility, invasion, and parasite transmission.

Endo-reduplication: replication of the nuclear genome in the absence of mitosis; results in elevated DNA content and polyploidy

Reversible protein phosphorylation: mechanism of activating or deactivating protein function by addition or removal of a phosphate group; coordinated by protein kinases (addition) and phosphatases (removal)

Molecular motor: responsible for intracellular transport of cargo, organelles, and vesicles along microtubules; examples include dyneins, kinesins, and myosins

2. SEXUAL DIFFERENTIATION AND PROLIFERATION

2.1. Atypical Mitosis and Axoneme Assembly During Male and Female Gamete Development: Ready for Fertilization

Upon ingestion by the mosquito in a blood meal, gametocytes are activated by environmental factors, including a drop in temperature (to approximately 20–25°C), a rise in pH (13), and the presence of the mosquito-derived metabolic intermediate xanthurenic acid (XA) (12) (**Figure 2*a*,*b***). Within seconds a tetrad of kinetosomes forms within the microgametocyte cytoplasm (143), resulting in microtubule-organizing centers (MTOCs) and kinetochores (115) that serve as anchors for mitotic spindles and the growth of axonemes (35). Within 12 min the microgametocyte has undergone three rounds of endomitotic DNA replication, increasing its DNA content from 1N to 8N (144). Subsequent karyokinesis and atypical cytokinesis produce eight microgametes that egress from the parasite body (a process termed exflagellation), leaving behind both residual nuclear and cytoplasmic masses (114) (**Figure 2*a***). Live-cell imaging of

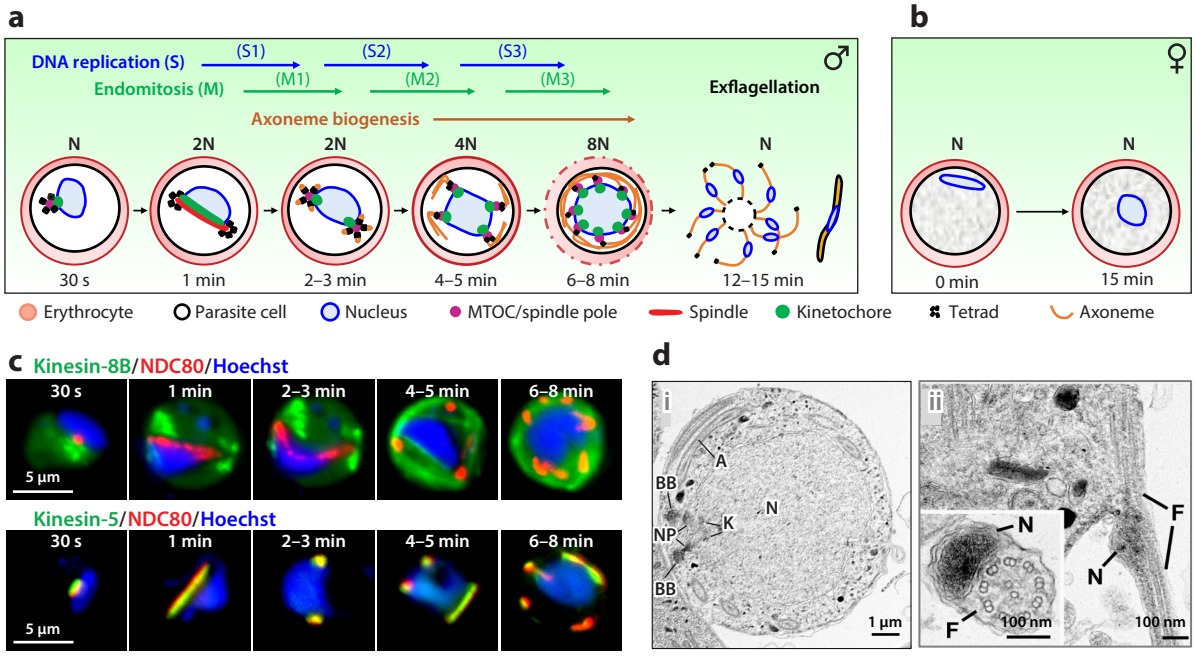

Figure 2

Events during male and female gametogenesis. (*a*) Schematic showing the process of atypical, closed mitosis during male gametogenesis within the mosquito gut. The process involves three rounds of DNA replication (S1, S2, S3) and mitotic division (M1, M2, M3), producing an octoploid nucleus (8N). Within 30 s of gametocyte activation, the tetrad of the basal body and microtubule-organizing center (MTOC) are observed close to the nucleus, along with the start of DNA replication. During the process, the tetrad of the basal body duplicates and then the tetrads move apart from each other in the cytoplasm. The axoneme starts assembling on these basal bodies and matures by 10–12 min after activation. At the end of these events, exflagellation produces eight haploid male gametes. (*b*) Schematic of female gametogenesis that shows no marked morphological changes except for a rounder and more condensed nucleus. One female gamete is formed from one female gametocyte. (*c*) Live-cell imaging of dual fluorescently (GFP/mCherry) tagged lines illustrating basal body (kinesin-8B), axoneme (kinesin-8B), kinetochore (NDC80), and spindle (kinesin-5) dynamics during male gametogenesis. Kinesin-8B is a basal body marker that initially localizes on the tetrad of the basal body and later moves onto the developing axonemes. NDC80 is a kinetochore marker that does not colocalize with kinesin-8B but overlaps with kinesin-5, located on spindle poles/MTOCs and spindles. Images adapted with permission from Reference 143. (*d*) Electron micrograph of a male gametocyte illustrating (*i*) a large central nucleus (N), nuclear poles (NP), basal bodies (BB), kinetochores (K), and an axoneme (A). (*ii*) Exflagellating male gametocyte with attached flagellum (F) and nucleus (N). (*Inset*) A cross section of a microgamete showing a flagellum with the classical 9+2 arrangement of microtubules and nucleus.

Microtubule-organizing center (MTOC): organizes flagella and the mitotic and meiotic spindle apparatuses during cell division

fluorescently tagged proteins, including kinesin-8B, NDC80, and kinesin-5, has revealed unique aspects of male gametogenesis. Kinesin-8B is a basal body marker that is localized on tetrads of kinetosomes and assists in axoneme assembly (**Figure 2*c***), while NDC80 is a kinetochore marker for chromosome segregation (**Figure 2*c***). Kinesin-5 is located on spindles, and fluorescence microscopy has demonstrated its location relative to kinetochores (NDC80) (**Figure 2*c***). Electron microscopy has been the gold standard for ultrastructural studies of the subcellular location of cell organelles. The basal body, nuclear pole, and kinetochore are evident in electron micrographs, complementing the live-cell imaging. The classical 9+2 arrangement of microtubules in flagella can be clearly seen in electron micrographs, but flagellum formation in *Plasmodium* is unique: All flagellum constituents are assembled in the cytoplasm of the male gametocyte, which lacks an intraflagellar transport (IFT) system (**Figure 2*d***). This lack of IFT may explain the inefficiency of the process, in which many incompletely formed axonemes are observed.

Male gametogony is orchestrated by several proteins to facilitate a rapid and highly coordinated sequence of events. Transient and reversible protein phosphorylation is key to this process (discussed in more detail in Section 2.2), beginning in *P. falciparum* with activation of guanylyl cyclases and phospholipase C (PLC) by XA, a rise in cyclic GMP (cGMP), and subsequent activation of protein kinase G (PKG) (77). In *P. berghei*, XA promotes activation of phosphoinositide (PI)-PLC and concomitant hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] (95), along with a rapid rise of calcium from both internal and external stores. Calcium-dependent protein kinase 4 (CDPK4) then initiates DNA replication (12, 63) and, along with two additional key proteins—the armadillo-repeat motif (ARM) protein PF16 and the spindle assembly–related protein SAS6—regulates efficient axoneme assembly (76, 119). Progression through gametogony occurs via CDPK1 (10, 106), with mitogen-activated protein kinase 2 (MAP2) and serine-arginine-related protein kinase (SRPK) regulating axoneme motility and subsequent cytokinesis (96, 122, 123) to produce eight microgametes. Additional key proteins in cytokinesis are cell division cycle 20 (CDC20) and anaphase-promoting complex/cyclosome (APC/C) subunit APC3, with deletion of *cdc20* and conditional knockdown of *apc3* resulting in loss of chromatin condensation at the nuclear spindle/kinetochore stage (42, 129). The metallo-dependent protein phosphatase PPM1, cyclin-dependent kinase (CDK)-related kinase 5 (CRK5), and Ca^{2+}-dependent calcineurin A are also involved in exflagellation (9, 45, 91); however, the precise timing of their activity is unknown.

When DNA replication is completed, the axonemes become motile and exflagellation occurs by the egress of basal bodies from the main cellular body, driven by the cytoskeletal actin-II (24) and the microtubule-based motor kinesin-8B (143). After detachment, PF16 regulates microgamete motility (119) until it encounters and attaches to a female gamete, mediated by the male-specific 6-cysteine proteins P48/45 and P230 (126) and the female-specific 6-cysteine protein, P47 (128). Subsequent gamete fusion is mediated by the plant-sterility gene *HAP2/GCS1* (71, 83) and the histone chaperone facilitates chromatin transcription (FACT) (66).

Female gametocyte activation by exposure to mosquito-derived factors in the mosquito midgut is similar to that of the male, resulting in PKG activation, rounding up of the cell, and emergence of the gamete from the erythrocyte (**Figure 2*b***) mediated by secretion of several female-specific proteins located in osmiophilic bodies [including PfG377, male development gene 1 protein (MDV1), and plasmodial perforin-like protein 2 (PPLP2)], which have a biolytic function (3, 92, 137). Development of the female gamete is accompanied by translational repression of hundreds of proteins in preparation for the zygote-to-ookinete transition. Over 50% of the macrogametocyte transcriptome is associated with two ribonucleoprotein complex proteins: the DDX6-class RNA helicase development of zygote inhibited (DOZI) and the Sm-like factor homolog of worm CAR-I and fly Trailer Hitch (CITH) (41, 72, 73); deletion of the genes for these two proteins results in complete ablation of zygote development and destabilization of hundreds of transcripts. In addition, the apetala2 (AP2) family transcription factor AP2-O3 (apetala-2 in ookinetes 3) acts as a translational repressor in female gametocytes, with mutant lines failing to differentiate into fertile macrogametes and undergoing developmental arrest at fertilization or in early development after fertilization (68).

2.2. Unique Meiotic Processes During Zygote and Ookinete Differentiation: Preparing for Invasion

Differentiation of the zygote results in the development of apical polarity and elongation to form the mature motile ookinete. Development is highly coordinated and accompanied by distinct spatiotemporal expression of key proteins after fertilization, beginning with CDPK1-mediated release of maternal transcript repression (106). The zygote is the stage in which meiosis begins, with

Kinetochore: large protein complex that assembles on the centromere; organizes attachment of spindle microtubules to chromosomes during mitosis

Mitotic spindle: cytoskeletal structure that separates sister chromatids between daughter cells during mitosis

Axoneme: microtubule-based cytoskeletal structure that forms the core of a flagellum; organized in a 9+2 configuration

Exflagellation: spontaneous formation and release of flagellated male sex cells by activated *Plasmodium* microgametocytes

NDC80: outer kinetochore complex that attaches the centromere of chromosomes to microtubules of the mitotic spindle

Gametogenesis/ gametogony: process by which *Plasmodium* male and female sex cells are produced after activation of gametocytes in the mosquito vector

Basal body: formed at the base of the flagellum and serves as a nucleation site for the developing axoneme microtubules

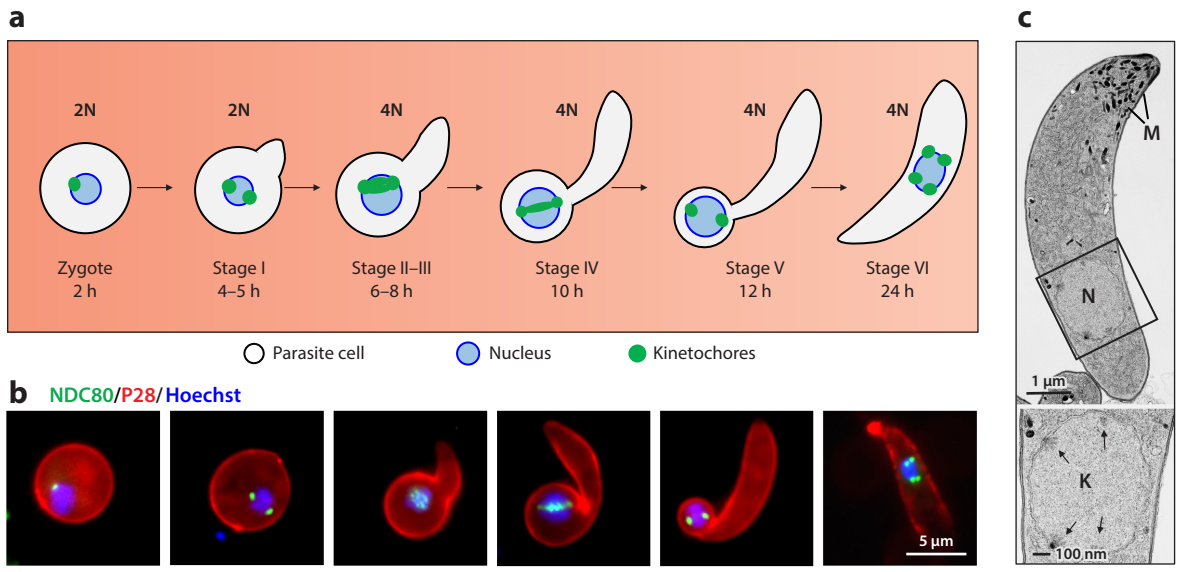

Figure 3

Zygote-to-ookinete differentiation and chromosome segregation dynamics using NDC80 as a kinetochore marker. (*a*) Schematic showing developmental stages of zygote transformation into a crescent-shaped ookinete within 24 h and illustrating kinetochore dynamics during meiosis. (*b*) Live-cell imaging showing the location of the kinetochore marker NDC80 (*green*) during various stages of ookinete development. P28 (*red*) is used as a surface marker. (*c*) (*Top*) Electron micrograph of an ookinete showing the nucleus (N) and the micronemes (M) at the apical end. (*Bottom*) Enlarged image of the nucleus showing four electron-dense kinetochores (K), similar to NDC80 (*green*) in panel *b*. Panels *b* and *c* adapted from Reference 144 with permission.

an initial DNA replication from 2N to 4N over a period of 4 h (**Figure 3*a***), and it is probably completed in the oocyst with the production of haploid sporozoites (**Figure 4*a***). Genetic recombination occurs during stages II–III (6–8 h) of ookinete development, with active involvement of spindle formation and chromosome segregation (**Figure 3*b***). In mature ookinetes, four sets of kinetochores are visible (four foci of NDC80 and four electron-dense regions in the nucleus), representing the 4N genome (**Figure 3*c***). Ookinete differentiation is regulated by two protein kinases—NIMA (never-in-mitosis gene A)-related kinase 2 (NEK2) and NEK4—and potentially the metallo-dependent protein phosphatase PPM2 (45, 98, 99). The parasite undergoes substantial morphological transformation, with formation of the inner membrane complex (IMC) and establishment of apical polarity driven by two IMC subcompartment proteins (ISP1 and ISP3) and by myosin B, SAS6L, and protein phosphatase 1 (PP1) (93, 130, 133, 141, 145). Establishment of apical polarity is followed over the next 24 h by further morphogenesis and elongation to produce the crescent-shaped motile ookinete. Ookinete development comprises six morphologically distinct stages (109). In stage I, the initial spherical body is indistinguishable from the female gamete or zygote; in stages II–V, a protuberance from the main body (which still contains the nucleus) grows and elongates; and by stage VI, the nucleus has migrated to the center of the mature, fully differentiated crescent form. Evidence for the identity of key proteins involved in the elongation stages is scarce, but PPM2 and protein phosphatase containing Kelch-like domains (PPKL) have been shown to be essential for progression from stage II to stage IV (44).

The mature ookinete has numerous apical secretory organelles termed micronemes, the contents of which are essential for its motility and migration through the midgut epithelium. The microneme secretome contains 345 proteins (65), some of which likely have vesicle trafficking and

Microtubules: polymers of tubulin forming part of the cytoskeleton and involved in numerous cellular processes (including mitosis, cell motility, and intracellular transport)

Intraflagellar transport (IFT): bidirectional motility along axonemal microtubules toward formation and maintenance of eukaryotic flagella; absent in *Plasmodium*

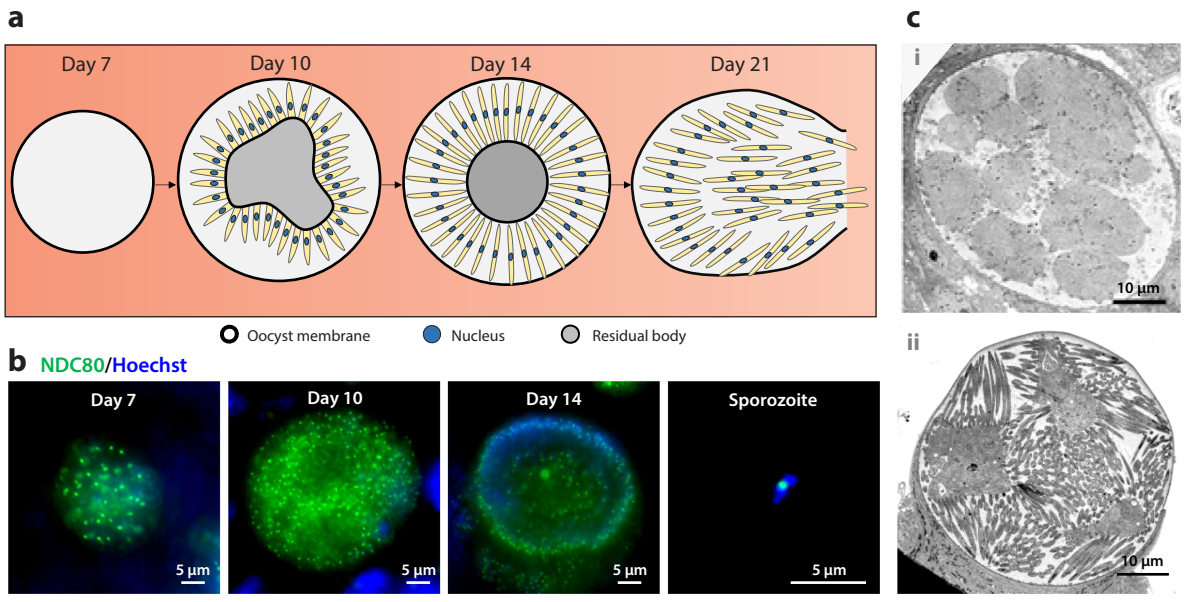

Figure 4

Proliferative stages of oocyst development and sporogony. (*a*) Oocyst proliferation and sporozoite formation. Oocysts burst after maturation (day 21), releasing sporozoites that migrate to the salivary glands. (*b*) Live-cell imaging showing the location of the kinetochore marker NDC80 (*green*) during various stages of oocyst development and in a sporozoite. (*c*) Electron micrographs of oocysts illustrating (*i*) budding and (*ii*) sporozoite formation. Figure adapted from Reference 144 with permission.

signaling functions required for micronemal secretory pathways. Motility of the mature ookinete is driven by the glideosome located between the parasite plasma membrane and the IMC (62), initiated by ISP1-anchored polarization of the guanylate cyclase β (GCβ)/CDC50A complex and cGMP signaling (15, 34, 81). Gene deletion studies have highlighted the downstream essentiality of CDPK3 and circumsporozoite- and thrombospondin-related anonymous protein (TRAP)-related protein (CTRP); their absence results in significantly reduced gliding motility and complete ablation of oocyst formation (26, 110, 140). The ookinete migrates through the mosquito midgut epithelium, facilitated by secretion of three PPLPs that contain a membrane attack complex/perforin (MACPF) domain. Deletion of the PPLP3 [also known as membrane attack ookinete protein (MAOP)], PPLP4, or PPLP5 gene results in complete ablation of oocyst development (25, 28, 56, 136). In the case of PPLP4, this phenotype can be circumvented by injection of cultured ookinetes directly into the mosquito thorax (25). Additional proteins identified through gene deletion studies as important for ookinete invasion and traversal are an ancient bacterial *Shewanella*-like protein phosphatase (SHLP1) (89), cell-traversal protein for ookinetes and sporozoites (CelTOS) (60), and secreted ookinete adhesive protein (SOAP) (27).

3. ASEXUAL PROLIFERATION AND TRANSMISSION

3.1. Oocyst and Sporozoite Development—Endomitosis and Invasion: Gearing for Transmission

The zygote-to-ookinete transition and oocyst development are significant bottlenecks in the parasite life cycle. From the thousands of gametocytes ingested, only 50 to 100 will develop into mature ookinetes. Of these, only 10% will complete oocyst development (38, 135), because most (>80%)

ookinetes are destroyed (22, 107). Oocyst development is the longest phase of the life cycle, lasting approximately 14 days and culminating in release of mature sporozoites that invade the mosquito salivary glands after 21 days, ready for transmission during the next blood meal (**Figure 4*a***).

Endomitosis: division of chromosomes in the absence of nuclear membrane dissolution and concomitant cytokinesis

Following traversal of the peritrophic matrix and the midgut epithelium, an ookinete attaches to the basal lamina, triggering differentiation to an oocyst (113), which is thought to proceed via interaction of P25/28, CTRP, and SOAP with laminin (6). Over the next two to three weeks, the parasite genome undergoes multiple endomitotic replication (during sporogony), and the oocyst increases in size to a diameter of 50–60 μm (**Figure 4**). The kinetochore marker NDC80 has been used to follow endomitosis, revealing multiple foci adjacent to the nuclear DNA (**Figure 4*b***). Upon initiation of sporogony, sporoblast retraction of the parasite plasma membrane produces deep invaginations forming cytoplasmic lobes but with no clear cytokinesis, resulting in a huge increase in parasite plasma membrane surface area and allowing hundreds of sporozoites to be produced (**Figure 4*c***). This is similar to what is observed in large schizonts of *Eimeria bovis* (48).

Numerous proteins have been implicated in sporogony (5), but key to its completion are acquisition of nutrients from the host, evasion of the mosquito immune system (reviewed in 117), and regulation of DNA replication and metabolic processes in the parasite (118). We have shown that the single *Plasmodium* P-type cyclin (CYC3) is an essential mediator of endomitosis in the developing oocyst (101); its absence results in complete lack of retraction of the plasmalemma to form sporoblasts. Recently, we showed that deletion of genes encoding the DNA repair protein meiotic recombination 11 (MRE11) and 3-hydroxyacyl-CoA dehydratase (DEH) [a protein involved in fatty acid synthesis (FAS)] results in degenerative oocysts and a complete lack of sporogony (43, 46). This phenotype is similar to that observed in other functional studies of *Plasmodium* FAS in which deletion of components of the FAS II pathway ablates sporogony and results in degenerative oocysts (118, 127). The protein kinases GAK and PK7, as well as PPM5, have also been implicated in oocyst development (45, 123).

At 16–21 days after the blood meal, mature sporozoites egress from the oocyst into the hemocoel, mediated by the putative cysteine protease egress cysteine protease 1 (ECP1) (4) and circumsporozoite protein (CSP) (132). Then they migrate to the salivary glands, where they attach to and invade the tissue via TRAP and the TRAP-like protein upregulated in oocyst sporozoites 3 (UOS3) (79), cysteine-repeat modular proteins (CRMPs) (124), and merozoite adhesive erythrocytic binding protein (MAEBL) (61). For more in-depth reviews of sporozoite development and biology, see References 5, 32, and 59.

4. MOLECULAR AND FUNCTIONAL SCREENS—INSIGHTS INTO CONTROL OF PARASITE DEVELOPMENT

Molecular and genome-wide functional screens have been vital in elucidating the key players in malaria parasite development in both vertebrate host and mosquito vector. Two genome-wide functional screens showed that over half the *Plasmodium* genome is essential for blood stage development (16), and of the genes that are dispensable at this stage, over 450 are required for efficient transmission through the mosquito (118). Development of the parasite in the mosquito is largely governed by two major processes: regulation of gene expression and reversible protein phosphorylation. Here, we discuss a few key in-depth studies that have elucidated the role of transcription factors; the importance of posttranslational modification by reversible protein phosphorylation; and the function of motor proteins in regulating parasite development, proliferation, differentiation, and invasion, with a focus on sexual and transmission stages within the mosquito vector (**Table 1**).

Table 1 Molecules regulating parasite proliferation, invasion, and development in the mosquito vector

Molecules	Gametocyte		Ookinete	Oocyst	Sporozoite
	Female	Male			
Transcription factors	Unknown	Unknown	AP2-O, AP2-O2, AP2-O3, AP2-O4, AP2-O5	None	AP2-SP, AP2-SP2, AP2-SP3
Protein kinases	NEK2, NEK4	CDPK4, CRK5, SRPK1, MAP2	CDPK3, GAK, PK7	CDLK, CDPK6, PK7	PbKIN
Protein phosphatases	Unknown	PPM1	PPM2, PPKL, SHLP1	PPM5	Unknown
Kinesins	Unknown	Kinesin-8B, kinesin-13, kinesin-15	Kinesin-13, kinesin-20	Kinesin-8X	Kinesin-5
Myosins	Unknown	Unknown	Unknown	Unknown	MyoE
Cyclins	Unknown	Unknown	Unknown	CYC3	Unknown
Condensins	Unknown	Unknown	Unknown	SMC2, SMC4	Unknown
Anaphase-promoting complex	Unknown	CDC20, APC3	Unknown	Unknown	Unknown
Others	P47	Actin-II, SAS6, GEST, HAP2, PF16, P48/45, P230, FACT	DOZI, CITH, CAX, AP2-O, CelTOS, TRAP, PDEδ	GEX, DMC1, MISFIT, C-CAP, DEH, MRE11	LAPs, CDLK, CelTOS, SPECT, SPECT2, TRAP, CSP

Abbreviations: AP2-O, apetala2 in ookinetes; AP2-SP, apetala2 in sporozoites; APC, anaphase-promoting complex; CDC, cell division cycle; CDPK, calcium-dependent protein kinase; CelTOS, cell-traversal protein for ookinetes and sporozoites; CITH, CAR-I and fly Trailer Hitch; CRK, cyclin-dependent kinase (CDK)-related kinase; CSP, circumsporozoite protein; DEH, 3-hydroxyacyl-CoA dehydratase; DOZI, development of zygote inhibited; MAP, mitogen-activated protein; MRE, meiotic recombination; NEK, NIMA (never-in-mitosis gene A)-related kinase; PK, protein kinase; PPKL, protein phosphatase containing Kelch-like domains; SHLP, *Shewanella*-like protein phosphatase; SMC, structural maintenance of chromosomes; SRPK, serine-arginine-related protein kinase.

4.1. Transcription Factors Controlling Development

Malaria parasites are unique in that they possess only a small number of regulatory genes compared with other eukaryotic organisms. Here, we describe the major transcription factors that regulate *Plasmodium* development in the mosquito.

4.1.1. The ApiAP2 family. Despite its complex life cycle, *Plasmodium* has few transcription factors compared with other eukaryotes (1, 97). The majority are encoded by a single family comprising 26–27 members (depending on the *Plasmodium* species) known as the AP2 domain–containing ApiAP2 protein family (8). The ApiAP2 family domain is found in proteins encoded by all Apicomplexa investigated to date (87), and these proteins act as both repressors and activators of transcription (69) (**Supplemental Table 1**); they are homologous to the plant AP2/ethylene response factor (ERF) family of DNA-binding proteins (100).

Supplemental Material >

Initial functional studies of *Plasmodium* ApiAP2 members revealed their role in stage-specific regulation of gene transcription (54, 138, 139), with AP2-G and AP2-G2 described as master regulators of sexual commitment (57, 116). Yuda et al. (139) performed a systematic investigation of predicted AP2 factors expressed only in *P. berghei* mosquito stages, revealing that a protein they designated as AP2 in ookinetes (AP2-O) is translationally repressed by DOZI (72) and essential for ookinete morphogenesis. AP2-O directly regulates expression of the genes for 15 microneme- or cell surface–associated proteins by binding to a conserved (17) *cis*-acting control element—TAGCTA—within 1 kb upstream of the open reading frame. This finding was confirmed

by Kaneko et al. (58), who discovered that AP2-O directly regulates approximately 10% of the parasite genome. It has also been demonstrated that *P. berghei* AP2 in sporozoites (AP2-SP) is essential for sporogony, shows nuclear expression in late oocyst and sporozoite stages (138), and binds the *cis*-element GCATGCA upstream of sporozoite-specific genes (23). A genome-wide knockout screen of all *P. berghei* ApiAP2 family members confirmed that AP2-O and AP2-SP proteins are not essential in blood stages and identified three additional AP2-O genes (encoding AP2-O2 through AP2-O4) that are critical to the formation of infectious ookinetes and another two AP2-SP genes (encoding AP2-SP2 and AP2-SP3) required for sporogony (80). Similar findings were also reported for a CRISPR-Cas9-based study in *Plasmodium yoelii* (148), which revealed an additional AP2-O gene that regulates ookinete motility and is essential for oocyst development (encoding a protein designated AP2-O5).

4.1.2. The Puf family. The Pumillo/FBF (Puf) family of transcription factors is evolutionarily conserved in eukaryotes and characterized by a Puf RNA-binding domain; these proteins either translationally repress mRNAs or induce their degradation (94). *Plasmodium* encodes two Puf members (Puf1 and Puf2) (21), both of which are expressed in gametocytes and have their highest expression in sporozoites (47, 67). Targeted deletion of both genes in *P. berghei* revealed a functional redundancy during mosquito stages. However, there was a significant reduction of mouse infectivity in the absence of Puf2 (85) as a result of premature expression in sporozoites of genes that are usually repressed until development in the liver, transforming salivary gland sporozoites into early liver stage forms (37, 85). *Puf2* deletion studies in *P. yoelii* confirmed this phenotype and suggested a key role in directly or indirectly maintaining the homeostasis of specific transcripts (70).

4.2. Reversible Protein Phosphorylation Regulating Differentiation, Motility, and Invasion

Reversible protein phosphorylation catalyzed by protein kinases and protein phosphatases is a ubiquitous and highly conserved regulatory process that governs many eukaryotic and prokaryotic cellular pathways (20). Genome-wide functional screens and molecular characterization of the *Plasmodium* kinome and phosphatome have revealed that approximately a third of protein kinases and protein phosphatases have functions specific to the mosquito stages. A seminal study highlighted the rapid and highly temporal nature of gametogenesis, with distinct cell cycle events and simultaneous phosphoregulation at specific time points (53). Within 1 min of male gametocyte activation, most phosphoregulation governs microtubule-based complexes and movement, whereas phosphorylation implicated in DNA replication was not detected until 5 min after activation. This study suggests a coordinated, spatiotemporal interplay between protein kinase and protein phosphatase activities, which potentially could be manipulated therapeutically using targeted inhibition.

4.2.1. Protein kinases: essential regulators of parasite transmission. The *Plasmodium* kinome contains between 60 and 90 protein kinases depending on the species, accounting for ~2% of its genome (134). Systematic functional analysis in *P. berghei* identified 12 mutants with a mosquito stage phenotype (123) (**Supplemental Table 2**). Further genetic studies have examined the role of these protein kinases: In male gametogenesis, CDPK4 initiates DNA replication and axoneme assembly in the pre-replicative phase within 10 s of activation, whereas MAP2 and SRPK regulate subsequent axoneme motility and cytokinesis (42, 122). Absence of NEK2 and NEK4 genes—which encode two of the four members of the NIMA family—ablates the single

Supplemental Material >

round of meiotic genome replication in the zygote (98, 99). CPDK3 is required for ookinete gliding (110), and although GAK [postulated to have a role in clathrin-mediated vesicle trafficking (103, 108)] and PK7 are involved in ookinete development (123), their exact function is still unknown. A study by Gomes-Santos et al. (36) using *Plasmo*GEM technology (123) identified an additional four protein kinases (TKL1, TKL3, GSK3, and PBANKA_1305200) important for parasite development in the mosquito and transmission (118) (**Supplemental Table 2**).

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CRK5 is a critical mitotic regulator during male gametogony, phosphorylating canonical CDK motifs of pre-replicative complex components (9). Disruption of CDPK1 and CDPK2 genes using CRISPR-Cas9 resulted in ablation of male gamete exflagellation (10, 11), with severe retardation of female gametocyte rounding also observed in the absence of CDPK1 (10). Finally, depletion of *P. falciparum* CRK4 during sexual development resulted in greatly diminished oocyst numbers, with necrotic parasites reminiscent of dead ookinetes (33).

4.2.2. Protein phosphatases: key orchestrators of ookinete development and invasion. A genome-wide analysis identified six protein phosphatases crucial for transmission (45) (**Supplemental Table 2**). Two protein phosphatases are essential for gametocyte development and gametogony: There is no exflagellation in PPM1 mutants [also suggested by Invergo et al. (53)], and PPM2 [an unusual chimeric PP2C member (75)] regulates the male-to-female gamete sex ratio. Calcineurin A was judged likely essential on the basis of results from a gene-deletion strategy, and an auxin-inducible degron conditional knockdown strategy showed that it is important for male gametogony (91). More recently, PP1 was shown to be a key regulator of chromosome segregation during mitotic exit in male gametogony (145), with conditional PP1 gene knockdown in gametocytes resulting in loss of exflagellation and structural defects similar to those observed in CRK5 mutants (9). The cyclic enrichment of PP1 at the kinetochore during onset of and exit from mitosis is consistent with a key role at this stage. PPM5 mutants have structural defects similar to those of SHLP1 mutants, with retarded oocyst development, and no sporogony or transmission. The chimeric PPM2 appears to be involved in sex determination and zygote development: Its knockdown resulted in a male-to-female gametocyte ratio of 1:1 (instead of the usual 1:3) and, in the differentiating ookinete, a highly variable DNA content in meiosis, cytoskeletal deformities, and an absence of micronemes. A recent study highlighted 10 genes with sex-specific roles in gametocytogenesis (102), and it will be interesting to see whether PPM2 targets any of the proteins they encode.

Detailed functional studies of protein phosphatases during ookinete differentiation, motility, and invasion showed that a PPKL mutant had mislocalized cytoskeletal proteins and the apical microtubules were dissociated from the IMC, resulting in nonmotile, abnormally shaped ookinetes arrested at development stage III and unable to invade mosquito midgut epithelium (44, 90). An SHLP1 mutant produced reduced numbers of motile ookinetes, with ablated oocyst development (89). However, this phenotype was bypassed by parasite injection into the hemocoel—suggesting that SHLP1 is essential only for midgut invasion. An ultrastructure analysis revealed defects in microneme development, with 80–90% of SHLP1 mutant cells having no, or severely reduced numbers of, micronemes, consistent with an essential role for SHLP1 in invasion.

4.3. Molecular Motors Controlling Cell Differentiation, Division, and Invasion

Parasite gliding motility, invasion, and proliferation within the mosquito are driven by the coordinated action of molecular motors. Three molecular motor proteins families are known: myosins, which move actin filaments to generate force (52), and kinesins and dyneins, which work

on microtubules (105). Systematic analyses of the location and role of myosins and kinesins have highlighted a diverse range of spatiotemporal expression and function.

4.3.1. Myosin family. Myosins perform a variety of functions (49); in *Plasmodium* they are important for differentiation, host interactions, and cell invasion (78). The *Plasmodium* genome encodes six myosins, divided into three classes based on head domain sequence: class VI (MyoJ and MyoK), class XIV (MyoA, MyoB, and MyoE), and class XXII (MyoF). Class XIV myosins are largely restricted to the Apicomplexa and certain ciliates (84); MyoF, MyoJ, and MyoK are conserved between *Plasmodium* and *Toxoplasma* (30).

Supplemental Material >

MyoA is a vital component of the glideosome and is required for gliding motility and host cell invasion (14, 50, 78, 106, 111). There are distinct patterns of expression, subcellular location, and function of the other myosins in the mosquito stages (131) (**Supplemental Table 3**). Deletion of the MyoE gene had minor effects on the number and motility of salivary gland sporozoites; deletion of the MyoB or MyoJ gene had no apparent consequence, suggesting that both are redundant (131). Analysis of myosin expression and location within the mosquito revealed that class XIV myosins are specific to ookinete, oocyst, and sporozoite stages. In the ookinete, MyoA is associated with the surface pellicle (40) and colocalized with ISP1 (a marker of early apical end development), MyoB has a very discrete location at the apical end, and MyoE is located at the basal end. MyoF and MyoK are located at the apical end and at two foci within the ookinete nucleus, respectively. MyoJ is expressed only in oocysts.

4.3.2. Kinesin family: key drivers of spindle dynamics, chromosome segregation, and microtubule organization. Kinesins are molecular motors important in cell polarity, cytoskeleton-associated motility, mitotic spindle formation, and chromosome segregation during cell division (64). *P. berghei* kinesins fall broadly into three functional categories: spindle and chromosome segregation, axoneme assembly and flagellum formation, and microtubule organization (**Supplemental Table 3**). Kinesin-8X and kinesin-5 are located at the kinetochore throughout most of the life cycle, associate with both mitotic and meiotic spindles, and follow spindle dynamics throughout chromosome segregation during male gametogony (mitosis) and ookinete development (meiosis) (142, 147). Deletion of the kinesin-8X gene or the kinesin-5 gene results in a significant reduction in oocyst development or sporozoite numbers, respectively, suggesting crucial roles during endomitosis in sporogony.

The other kinesins are associated with axoneme assembly during male gametogony or pellicle formation in the ookinete. Three kinesins (kinesins-8B, -15, and -X4) are in the cytosol of male gametocytes (146), whereas kinesin-X3 and kinesin-20 are cytosolic during ookinete and sporozoite development. Deletion of kinesin-8B ablates exflagellation completely, with defective basal body and flagellum formation and randomly oriented doublet and single microtubules within the cytoplasm (143), whereas deletion of kinesin-15 significantly reduces the number of exflagellating male gametes (143, 146). Kinesin-20 is located at the developing apical protuberance from the main cell body of the zygote as a ringlike structure, suggesting a key role in ookinete elongation. This notion is supported by the bulbous shape of the mutants, which is due to defective microtubule elongation (146). Kinesin-13 is located in both the cytosol and nuclear compartments and is the only kinesin expressed throughout the life cycle. It associates with the kinetochore during chromosome segregation in proliferative stages and with axonemes in male gametocytes. Conditional knockdown of kinesin-13 significantly reduces numbers of male gametes, owing to defects in spindle formation and axoneme assembly. The small number of parasites with kinesin-13 mutations that do produce ookinetes all show defective microtubule organization and are unable to produce any oocysts.

5. OTHER MOLECULES AFFECTING PARASITE PROLIFERATION

5.1. Cyclins in Atypical Cell Proliferation

In eukaryotes, CDKs form complexes with their partner cyclins that govern cell cycle checkpoints and progression through cell cycle entry (G1/S) and mitotic transition (G2/M) (74, 125). *Plasmodium* species encode a small, unusual set of cyclins, only three of which are identifiable by sequence similarity: CYC1 and CYC4 (group III H and L cyclins, respectively) and CYC3 (a group II, P-type cyclin). Group I cyclin genes (involved in cell cycle progression) are completely absent, a common feature of all apicomplexans except *Cryptosporidium* (101). Functional studies have been focused on a group II P-type cyclin, CYC3. Live-cell imaging revealed a temporal pattern of CYC3-GFP expression during sporogony, and CYC3 gene deletion caused a significant inhibition of oocyst sporulation, with ablation of plasmalemma retraction, abnormal membrane reduplication, and defects in mitosis and cytokinesis (101). These findings suggest that *Plasmodium* CYC3 is involved in cell cycle progression, although its associated CRK partner and the timing of its function remain unknown.

5.2. Condensins and Chromosome Organization

Condensins form large, multisubunit protein complexes that play key roles in regulating chromosome condensation and segregation during cell division (51). Two condensin complexes exist in higher eukaryotes (condensin I and condensin II), whereas only one condensin complex is known in single-cell organisms such as *Schizosaccharomyces pombe* and *Escherichia coli* (39). Condensin I and condensin II share two common subunits belonging to the structural maintenance of chromosomes (SMC) ATPases (SMC2 and SMC4); however, each condensin is distinct since it interacts with one kleisin and two Heat protein subunits: Condensin I interacts with kleisin Iγ (CAP-H), HEAT IA (CAP-D2), and HEAT IB (CAP-G), whereas condensin II interacts with kleisin IIb (CAP-H2), HEAT IIA (CAP-D3), and HEAT IIB (CAP-G2) (86, 104).

All subunit components of the condensin I and condensin II complexes are encoded in the *P. berghei* and *P. falciparum* genomes (7, 88) and in other Apicomplexa, including *Cryptosporidium* (88, 121). The SMC2/SMC4 core subunits are expressed during each proliferative stage of the parasite life cycle, dispersed in the nucleus or at discrete foci adjacent to the DNA and localized to centromeres in male gametocytes. SMC2 and SMC4 are essential for asexual blood stage development (i.e., schizogony); therefore, the genes encoding them are refractory to deletion. However, conditional gene knockdown has revealed a key role for both during oocyst development: Their absence results in complete ablation of parasite transmission, with defects also observed during male gametogenesis (88). Currently, the function and location of other condensin I and condensin II subunit components are unknown.

5.3. The Anaphase-Promoting Complex/Cyclosome

The anaphase-promoting complex/cyclosome (APC/C) is a large, highly conserved, multisubunit E3 ubiquitin ligase that targets mitotic proteins involved in the metaphase-to-anaphase transition for degradation by the 26S proteasome (19, 82, 120). The APC/C has two major regulators in most eukaryotic systems: CDC20 and CDC20 homolog 1 (CDH1). Mammals have 14 additional subunits that serve mainly as adaptors (18, 82).

Plasmodium encodes a single CDC20/CDH1 homolog (42) and only three APC/C subunits: APC3 (also known as CDC27), APC10, and APC11 (29). CDC20 is critical for male gametogony, particularly cytokinesis, with deletion mutants undergoing DNA replication but showing severe defects in nuclear spindle kinetochore formation and complete ablation of exflagellation (42). A

promoter trap strategy (106) for APC3 revealed a gametogony phenotype similar to that of the CDC20 mutant, with loss of APC3 resulting in loss of chromosome condensation and exflagellation (129). The essential functions of the other APC components, APC10 and APC11, are unknown; however, none of the *Plasmodium* APC/C components forms a stable protein complex, suggesting that each acts independently during the cell cycle (129).

6. CONCLUSIONS

Genome-wide functional screens in *P. berghei* have been instrumental in identifying the key players involved throughout *Plasmodium* development in the mosquito vector. With the advent of new technologies at the cutting edge of cell biology and gene targeting, further advances are sure to help answer the outstanding questions and translate current findings to *P. falciparum*, with the ultimate aim of identifying targets for therapeutic drug development to significantly reduce the socioeconomic burden of this devastating disease.

SUMMARY POINTS

1. Various parasite developmental stages within the mosquito have different morphology and modes of movement and invade different niches.
2. Genome-wide functional screens have uncovered proteins that are critical for parasite development in the mosquito.
3. Members of the apetala2 (AP2) family of transcription factors are master regulators of sexual differentiation and maturation, and many have essential roles during ookinete and sporozoite development.
4. Reversible protein phosphorylation is a crucial driver of sexual development, with a third of *Plasmodium* protein kinases and protein phosphatases functioning specifically in mosquito stages.
5. Molecular motors including myosins and kinesins drive parasite differentiation, motility, and invasion. Only one myosin has a specific role in determining salivary gland sporozoite numbers.
6. All but one kinesin (kinesin-13) have essential functions exclusively during mosquito stages.
7. Cyclins and condensins drive cell cycle progression and chromosome condensation, and their absence severely affects oocyst development.

FUTURE ISSUES

1. Although the key functions of the *Plasmodium* kinome and phosphatome are known, what are their substrates and targets?
2. How is the microtubule-organizing center (MTOC) organized in *Plasmodium* in the absence of a centrosome? How does it coordinate chromosome segregation, axoneme assembly in male cells, and subpellicular apical polarity during zygote differentiation?
3. How are basal bodies and axonemes organized during male gametogony, and how are the nuclear and cytoplasmic compartments coordinated during flagellated male gamete

formation? How does cell division proceed so quickly in proliferative stages, and how are various cellular components (such as centrosomes, hemispindles, kinetochores, and basal bodies) arranged? Is the process geared toward speed over efficiency and accuracy?

4. What protein complexes are formed by *Plasmodium* cyclins and cyclin-dependent kinase (CDK)-related kinases (CRKs), and how do they control the atypical cell cycle that lacks a G2 phase? When do they function during mitosis or meiosis?
5. Where do all the DNA precursors necessary to go from 1N to 8N during male gametogony come from? How are origins of replication regulated during the three rounds of genome replication during male gametogony?
6. During meiosis, how does each of the four genomes ensure it has one copy of each chromosome?
7. When does the second reductive meiotic division occur to produce haploid sporozoites? It may be reasonable to suggest that a second reductive division occurs in the ookinete-to-oocyst transition, but is the asexual proliferation (sporulation) associated with the oocyst similar to that of other developmental stages?
8. Do other posttranslational modifications regulate *Plasmodium* development in the mosquito? The ubiquitin-proteosome system (UPS) and lipid posttranslational modifications (such as palmitoylation and *N*-myristoylation) are key in other eukaryotes; what roles do they play during parasite transmission?

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RELATED RESOURCES

Euk. Pathog. Vector Host Inform. Res. PlasmoDB. **https://plasmodb.org/plasmo/app.** Official database of the *Plasmodium falciparum* Genome-Sequencing Consortium

Janse CJ. RMgmDB. **https://www.pberghei.eu/index.php**. Database of genetically modified malaria parasites generated by labs worldwide

Plasmodium Genet. Modif. Proj. *Plasmo*GEM. Wellcome Trust Sanger Inst. **https://plasmogem.umu.se/pbgem/**