Cell, Vol. 121, 675–687, June 3, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.03.027

Proteome Analysis of Separated Male and Female Gametocytes Reveals Novel Sex-Specific *Plasmodium* Biology

Shahid M. Khan,[1,2] Blandine Franke-Fayard,[1] Gunnar R. Mair,[1] Edwin Lasonder,[2,3] Chris J. Janse,[1] Matthias Mann,[2,*] and Andrew P. Waters[1,*]

[1]Department of Parasitology
Leiden University Medical Centre
Leiden
The Netherlands

[2]Center for Experimental BioInformatics
Department of Biochemistry and Molecular Biology
University of Southern Denmark
Odense
Denmark

[3]Department of Molecular Biology
NCMLS (191)
University of Nijmegen
Nijmegen
The Netherlands

Summary

Gametocytes, the precursor cells of malaria-parasite gametes, circulate in the blood and are responsible for transmission from host to mosquito vector. The individual proteomes of male and female gametocytes were analyzed using mass spectrometry, following separation by flow sorting of transgenic parasites expressing green fluorescent protein, in a sex-specific manner. Promoter tagging in transgenic parasites confirmed the designation of stage and sex specificity of the proteins. The male proteome contained 36% (236 of 650) male-specific and the female proteome 19% (101 of 541) female-specific proteins, but they share only 69 proteins, emphasizing the diverged features of the sexes. Of all the malaria life-cycle stages analyzed, the male gametocyte has the most distinct proteome, containing many proteins involved in flagellar-based motility and rapid genome replication. By identification of gender-specific protein kinases and phosphatases and using targeted gene disruption of two kinases, new sex-specific regulatory pathways were defined.

Introduction

Unicellular *Plasmodium* parasites switch from haploid asexual proliferation to sexual development in the blood of a vertebrate host in order to transmit between the vertebrate and the mosquito host. These haploid sexually differentiated male and female cells, named gametocytes, are the precursor forms of the male and female gametes. Gametocytes circulate in the blood of the vertebrate host in a state of developmental arrest until triggered to transform into gametes by environmental cues associated with the mosquito midgut. Activation results in the emergence of both gametes from the red blood cell (RBC), followed in the male by rapid 3-fold replication of its genome and the generation of eight motile gametes, which must locate and fertilize female gametes all within 10–30 min. Fertilization results in the formation of a diploid zygote that undergoes meiosis and develops into a motile tetraploid form, the ookinete. The ookinete traverses the midgut wall, forming an oocyst on the hemolymph side of the midgut to establish the parasitic infection of the mosquito vector (for reviews, see Janse and Waters, 2004; Sinden et al., 2004). Orchestrating these rapid life-stage switches are regulatory proteins. The precise number of conserved kinases and phosphatases is unclear but currently constitutes ~2% of the genes of *Plasmodium*, and their role is believed to be associated with the initiation of most transitional processes in the parasite (Anamika et al., 2005; Ward et al., 2004). An essential, calcium-dependent protein kinase signaling pathway has been identified in gametocytes that controls both male gamete formation and zygote development (Billker et al., 2004), but further detail in gametocyte-specific signaling processes is currently unavailable.

The central role of sexual development in the transmission of *Plasmodium* makes the surface proteins of the sexual stages, such as the gametes and zygotes, attractive targets for development of strategies that prevent transmission. For example, immune responses against surface proteins of the sexual stages, principally through the action of antibodies, have successfully targeted events during sexual development, resulting in reduction of transmission capacity (Carter, 2001; Kaslow, 1997). Until large-scale genome and postgenome analyses of *Plasmodium* were initiated, only a few sex-specific surface proteins had been described, and the proteins involved in the processes of sexual differentiation were largely unknown (Janse and Waters, 2004). Early transcriptome and proteome analyses of gametocytes showed that the switch from asexual to sexual development involved a significant reprogramming of transcriptional activity, resulting in the specific expression or upregulation of as much as 25% of the 5500 genes in the *Plasmodium* genome during sexual and zygote development (Florens et al., 2002; Hayward et al., 2000; Lasonder et al., 2002; Le Roch et al., 2003). In these studies, no distinction could be made between proteins and processes that are specific to either the male or female cells because it has not been possible to separate and purify the sexes. Male (MG) and female gametocytes (FG) are distinct: the MG prepare for DNA replication, mitosis, and gamete production, whereas FG prepare for meiosis and postzygote growth in the mosquito midgut environment (Janse and Waters, 2004). The MG- and FG-specific proteomes might be expected to differ considerably, and their characterization is therefore essential for understanding sexual differentiation and may identify potential vaccine targets.

We report an effective method for separation and purification of both sexes of *Plasmodium* based on transgenic GFP-expressing parasites and flow cytometry.

*Correspondence: mann@bmb.sdu.dk (M.M.); waters@lumc.nl (A.P.W.)

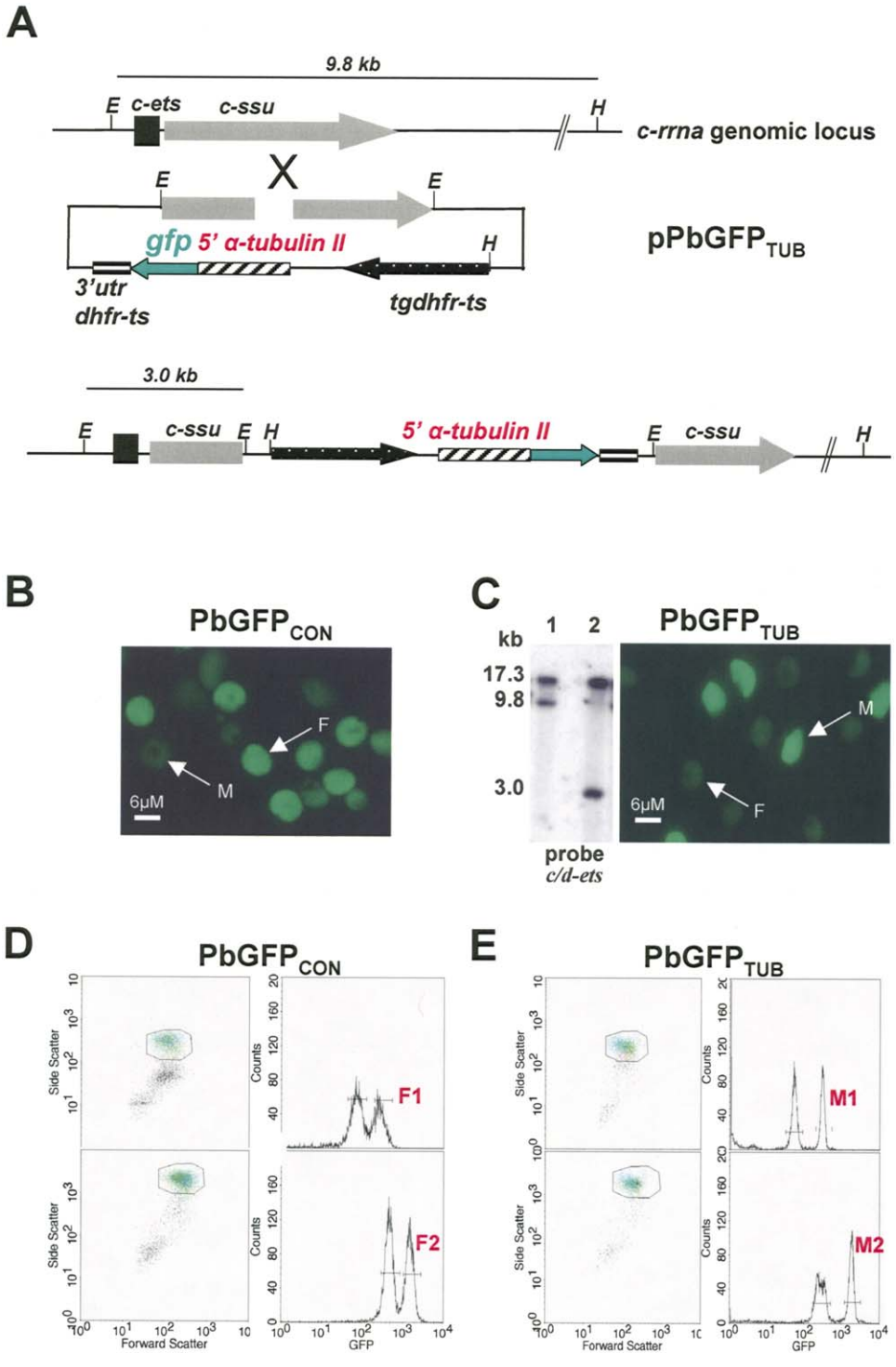


Figure 1. Generation and Analysis of Transgenic *P. berghei*, PbGFP$_{CON}$, and PbGFP$_{TUB}$ Lines Expressing GFP in Male and Female Gametocytes

(A) Schematic representation of the insertion plasmid used to generate the transgenic parasite line PbGFP$_{TUB}$ and of the target locus for genomic integration by single crossover, the small subunit (ssu) region of the *c-rrna* gene (before and after integration of the plasmid). The plasmid used contains the *gfp* gene under the control of the 5′UTR of the *P. berghei* α-*tubulin-II* gene in pPbGFP$_{TUB}$ and the *tgdhfr-ts* selection cassette. *E*: EcoRI; *H*: HindIII. The construct used for generation of pPbGFP$_{CON}$ has been described before (Franke-Fayard et al., 2004).

(B) Purified GFP-fluorescent mixed MG and FG of PbGFP$_{CON}$. The stronger fluorescent cells are FG (see also [D]).

(C) Correct integration of the insertion plasmid PbGFP$_{TUB}$, as shown by Southern analysis (left-hand panel). Genomic DNA was digested with EcoRI and HindIII and hybridized to the *c/d-ets* probe, which hybridizes to the external transcribed spacer region of both the *c-rrna* and the *d-rrna* gene unit (van Spaendonk et al., 2001). In DNA of wild-type parasites (left lane), this probe hybridizes to a 17 kb fragment (*d-rrna* gene

The proteomes of MG and FG were determined by highly sensitive and accurate liquid chromatography tandem mass spectrometry (LC-MS/MS) and confirmed and extended the anticipated divergent biology and gender-specific features of MG and FG. The gametocyte proteomes contained 29 protein kinases and phosphatases, many gender specific. Two sex-specific putative protein kinases, mitogen-activated protein kinase 2 (MAP2) and NIMA-related kinase (NEK4), were investigated using gene-disruption technology. MAP2 kinase is necessary for cell differentiation in male gametocytes after genome replication; the female-specific NEK4 has a postfertilization role and is required for meiosis to occur.

Results

Separation of Male and Female Gametocytes

The transgenic *P. berghei* PbGFP$_{con}$ constitutively expresses GFP under the control of the elongation factor 1 α (*eef1aa*) promoter region (Franke-Fayard et al., 2004). However, we subsequently observed that GFP fluorescence was markedly decreased in MG compared to FG. This can be attributed to the decreased ribosome and ribosome-associated-protein content of MG compared to FG (Janse and Waters, 2004). GFP$_{con}$FG show four times higher GFP fluorescence intensity than GFP$_{con}$MG (Figure 1). Also, GFP$_{con}$FG show the highest GFP expression, compared to all other (asexual) blood stages (Franke-Fayard et al., 2004). We sought to exploit this feature to separate the sexes using fluorescence-activated cell sorting (FACS). Flow sorting of purified, mixed gametocytes of PbGFP$_{con}$ led to the collection of highly pure female gametocytes that were sorted from the population with the highest fluorescence intensity (Figure 1D; right-hand peaks, F1 and F2). These samples consist of 99% females as determined by light microscopic analysis of the flow-sorted cells (data not shown).

However, the flow-sorted cells with the lower fluorescence intensity (Figure 1D; left-hand peak), presumed to be the MG, were contaminated with asexual and immature FG (10% contamination). This is because the starting mixed-gametocyte populations for flow sorting always contain a low level of contamination with asexual trophozoites/schizonts. In order to obtain a population of MG of comparable purity to the flow-sorted GFP$_{con}$FG, we therefore generated a second transgenic line (GFP$_{tub}$) containing *gfp* under control of the promoter of the male-specific α-*tubulin-II* gene (Rawlings et al., 1992). GFP$_{tub}$MG exhibit a much stronger GFP fluorescence intensity than FG (6:1 ratio; Figures 1C and 1E), corresponding to the high level of expression of α-tubulin-II in MG for the production of the eight flagella (Rawlings et al., 1992). As a consequence, we were able to obtain very pure MG parasites (>99%) from GFP$_{tub}$ by flow sorting as described above (Figure 1E; right-hand peaks, M1 and M2). Because asexual GFP$_{tub}$ blood stages show very low GFP fluorescence intensity compared to gametocytes, the flow-sorted FG from GFP$_{tub}$ (left-hand peaks in Figure 1E) can only be contaminated with young, developing MG, which are starting to express GFP. Since young MG cannot be distinguished morphologically or biochemically from FG (Janse and Waters, 2004), the precise level of contamination of GFP$_{tub}$FG with young MG is unknown.

In addition, a population of pure asexual blood stages (trophozoites/young and mature schizonts) from a nongametocyte-producer parasite (line HPE; Janse et al., 1989) was collected. Since this population does not contain any gametocytes, it served as a control for identification of asexual expressed blood-stage proteins in the proteomes of the flow-sorted, highly pure gametocytes and the contaminated gametocyte populations (see below). Finally, to compare our data to previous proteome studies using purified, mixed gametocytes, we collected and analyzed a mixed-gametocyte population that contained low-level contamination of asexual stages (see below).

Altogether, in four independent flow-sorting experiments each, we obtained two highly pure FG populations of GFP$_{con}$ and two highly pure MG populations of GFP$_{tub}$. The four less-pure preparations of flow-sorted gametocytes (GFP$_{con}$MG and GFP$_{tub}$FG) were also analyzed because they reveal the nature of their respective contamination (see below) and still provide information on the expression of sex-specific proteins. Including the asexual blood stages, we performed proteome analysis on ten samples, and in all flow-sorted populations of gametocytes, a fixed number of cells (1×10^7) was used.

Male- and Female-Specific Gametocyte Proteomes Determined by LC-MS/MS Analysis

Extracted trypsin-digested proteins of the ten samples were analyzed by LC-MS/MS essentially as described previously (Lasonder et al., 2002). The sequences of resulting tryptic peptides were searched against a combined database of predicted proteomes of *P. berghei* (generated from 3× genome coverage), mouse, and human proteins.

The purified, mixed gametocytes identified in total 13,801 peptides corresponding to 779 *P. berghei* proteins. In the flow-sorted, pure MG populations, 650 pro-

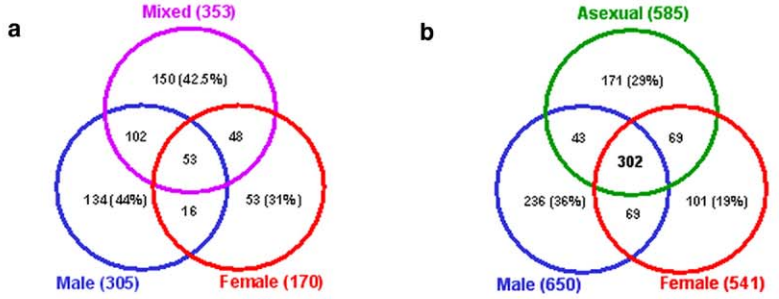
unit) and a 9.8kb fragment of the *c-rrna* gene unit, whereas a fragment of 3.0 kb of the *c-rrna* gene unit is detected after integration of the plasmids in the *c-rrna* unit (see [A]). In the right-hand panel, the purified, GFP-fluorescent mixed MG and FG of PbGFP$_{TUB}$ are shown; the MG show the most intense fluorescence (see also [E]).

(D and E) GFP fluorescent intensity of purified, mixed MG and FG in four experiments, as shown by FACS analysis. Left-hand panels: the dot-blot representations of the side and forward scatter of cells, showing the selection of cells that were used to measure fluorescence intensity during the sorting of MG and FG. Cells with a lower side/forward scatter that are excluded from the selection are platelets or cell debris. Right-hand panels: GFP fluorescence intensity of the MG and FG. In the fluorescence peaks, the gates that have been set to separate cells from the two peaks during cell sorting are shown. In PbGFP$_{CON}$, cells from the right-hand peaks (F1 and F2) are the highly purified FG populations, and in PbGFP$_{TUB}$, cells from the right-hand peaks (M1 and M2) are the highly purified MG populations.

A

				FEMALE				MALE			
Accession No	Protein Name	Pfal gene model	Mixed	1	2	3	4	1	2	3	4	Asexual
PB000172.01.0	merozoite surface protein 1, precursor, putative	PFI1475w	80				1		37		37	96
PB000779.00.0	hypothetical protein	PF14_0102	20						6		5	31
PB301475.00.0	rhoptry-associated protein, putative	PFE0075c	21			1			9		8	27
PB000108.03.0	cysteine protease, putative	PFB0355c	13						14		15	19
PB000887.00.0	conserved hypothetical protein	PF11_0268	9		3		1		6			16
PB001115.00.0	conserved hypothetical protein	PF10_0130	16		2		3		2			16
PB000198.02.0	conserved hypothetical protein	PF08_0091	6								1	14

B



C

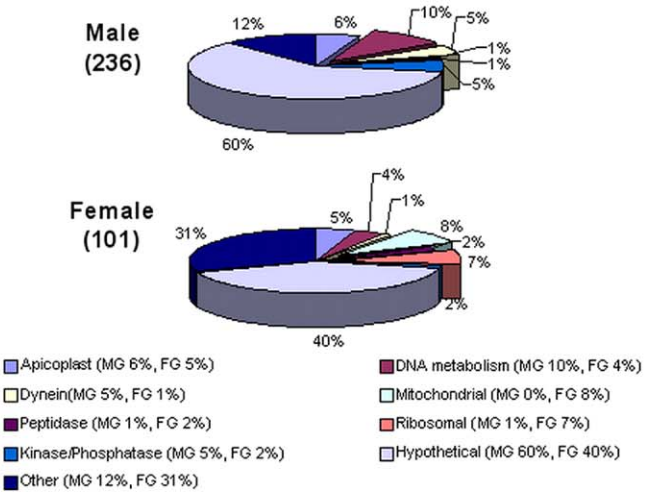


Figure 2. Distribution of Predicted Proteins from the *P. berghei* Proteomes of Asexual Blood Stages, Mixed Gametocytes, and Purified Female and Male Gametocytes

(A) Examples of the presence of proteins of asexual blood stages (number of unique peptides, i.e., those not sharing the same sequence) that show the contamination of the purified mixed gametocyte population (Mixed) and the sorted, contaminated FG and MG (columns 2 and 4) with asexual blood stages. These proteins are absent in the proteome of the highly pure MG and FG (columns 1 and 3). *P. berghei* gene models (Accession Number) as described in GeneDB (http://www.genedb.org/) and the *P. falciparum* orthologs (*P. fal* Gene Model) as in PlasmoDB (http://plasmodb.org/). Proteins shown are the seven proteins with the highest peptide hits in asexual blood stages that are present only in the contaminant proteomes and not in the proteomes of the highly pure male and females. For details on all contaminant proteins, see Table S8.

(B) Venn diagrams illustrating the overlap in proteins detected in the different proteomes. (Ba) Mixed gametocytes and the highly pure MG and FG. (Bb) Asexual blood stages and the highly pure MG and FG.

(C) Examples of differences in the distribution of proteins in MG and FG based on functional annotation, demonstrating the functional divergence between the sexes.

teins (identified by 8,470 sequenced peptides) were detected. In the pure FG, 541 proteins were similarly identified by 11,773 sequenced peptides. The two pure proteomes together overlapped with 73% of the proteins identified in the mixed-gametocyte proteome. We next sequenced the asexual, gametocyte-free sample,

resulting in 585 identified proteins. If any of these proteins of the asexual stages occurred in the other nine data sets of the gametocytes, they were removed to generate a gametocyte-specific set of proteins. This resulted in a mixed-gametocyte proteome of 353 proteins, a MG proteome of 305 proteins, and a FG proteome of 170 proteins (Figure 2B). For example, merozoite surface protein 1 is the most abundant protein found in asexual samples (judged by number of unique, identified peptides) and is also the most abundant protein in the contaminated $GFP_{con}MG$ and mixed-gametocyte samples. However, it is absent in the highly purified $GFP_{tub}MG$ and any FG population (Figure 2A). Furthermore, the absence of contaminating asexual stages in the $GFP_{con}FG$ and $GFP_{tub}MG$ samples allowed us to determine that 43 proteins were shared between MG and asexual and 69 between FG and asexual blood-stage parasites (Figure 2B). In total, 1104 different *P. berghei* proteins (including asexual and mixed-gametocyte samples) were identified, of which 1102 have a *P. falciparum* ortholog.

Comparison of the pure-gametocyte proteomes allowed identification of sex-specific and gametocyte-specific proteins. Interestingly, MG and FG share only 69 proteins, demonstrating that MG (236 proteins) and FG (101 proteins) are strikingly diverged with regard to sex-specific processes. This is perhaps not surprising in view of the different features and roles of MG and FG described above. Furthermore, MG are "terminally differentiated" cells with a (narrow) specific function and might be expected to contain a greater total number of stage-specific proteins than FG. A relatively high percentage of male proteins were annotated as hypothetical: 68%, compared to 54% in females (59% of predicted genes within the genome of *P. berghei* are designated as hypothetical). On average, 50% of the spectra generated for each sex-specific data set remained unassigned; a number of additional parasite proteins will likely be detected in retrospective analysis of these data as the *P. berghei* genome coverage and gene models are improved.

Protein annotation is chiefly based on sequence homology to previously characterized proteins. The function of most specific proteins discussed here is, in the absence of direct biochemical validation, putative.

Validation of the Sex Specificity of Proteins by Promoter Analysis

Encouragingly, most of the previously described, conserved, gametocyte-specific proteins are present in the MG and FG proteome data sets (Table S1). Furthermore, the proteome data correspond to the reported expression patterns in *Plasmodium* where known (Table S3). In particular, the proteome data of three members of the P48/45 family of surface proteins involved in fertilization, P47 (FG), P48/45 (FG and MG), and P230p (MG), correspond to sex-specific expression data using mono- or polyclonal antibodies (van Dijk et al., 2001; Williamson et al., 1995; M.R. van Dijk and B. Schaaik, personal communication). A total of 79 signal-peptide-containing proteins were predicted in the genome of *P. berghei*, of which 26 were identified in our study (including three MG-specific and six FG-specific; see Table S1). Further validation of the accuracy of MG and FG designation of the proteomes was achieved by analyzing the ability to drive the expression of GFP of the promoter region of ten representative genes encoding proteins identified in the various proteomes. Reporter plasmids were stably introduced into *P. berghei*, and GFP expression was monitored in blood stages (Table 1). For 9 of 10 promoters, GFP expression was in agreement with the prediction from the MG and FG proteome analysis. The single aberrant expression pattern (PB000119.00.0) is probably due to the short 5′ region available from the genome database. Importantly, apart from confirming the sex specificity of a number of FG and MG proteins, this analysis of promoter regions shows that sex-specific expression appears to be controlled by the 5′UTR/promoter and not by the 3′UTR or through posttranslational processes. In summary, the combination of subtraction of proteins present also in the asexual blood-stage proteome and promoter analysis confirmed the accuracy and specificity of the gametocyte-specific proteomes.

Sex- and Gender-Specific Features of Gametocyte Proteomes

A marked bias exists in the distribution of different classes of housekeeping proteins between MG and FG, which is reflective of their very different biological roles (Figure 2C). As a rough indication of protein amount, we compared the number of unique peptides identifying different protein classes in the four female and four male preparations. The FG proteome contains a greater abundance of ribosomal peptides (FG 1091: MG 535) and mitochondrial peptides (FG 770: MG 170) (see Tables S4 and S5, respectively). This is in accordance with a higher abundance of ribosomes (and endoplasmic reticulum) in FG (Janse and Waters, 2004) and also the increased number and/or complexity of their mitochondria. Unexpectedly, since in asexual stages the apicoplast is closely associated with the mitochondrion (Hopkins et al., 1999), apicoplast proteins are not FG biased (FG 595 peptides: MG 496; see Table S6).

MG expressed a greater proportion of stage-specific proteins compared to any *Plasmodium* life-cycle stage yet characterized (134 proteins, 44% of those characterized, cf. FG 53 proteins, 27%; Figure 2B). Two obviously distinct features of MG biology, genome replication and the formation of eight motile gametes, are reflected in the proteomes. Indeed, of 25 annotated proteins involved in DNA replication, 17 are very strongly represented in the MG proteome (MG 17 proteins, 606 peptides; FG 10 proteins, 46 peptides; see Table S7). The axoneme is exclusively associated with male gametes and is a structure that in a variety of organisms has been shown to comprise over 200 proteins (Inaba, 2003; Ostrowski et al., 2002). Examination of the proteomes revealed that axoneme-associated proteins are overwhelmingly represented in the MG proteome (21 of 32 annotated proteins, 578 peptides) compared with the FG proteome (3 proteins, 87 peptides). The MG bias in axoneme protein content is further emphasized if one subtracts the 82 of the 87 FG axoneme peptides that are derived from a single dynein heavy chain protein (PB000989.00.0; MAL7P1.162) that is unique to FG (Table 2).

Table 1. GFP Expression in Gametocytes and Asexual Blood Stages of Transgenic Parasites that Contain GFP under the Control of Promoters of Sex-Specific and Asexual Genes Predicted by the Proteome Data

			Proteome Data									GFP Expression			
			Female				Male								
Accession Number	Protein Name	Promoter (Size)	1	2	3	4	1	2	3	4	Asex	Fem	Male	Asex	Agreement
PB000652.01.0	**LCCL domain-containing protein CCP2 (female)**	**1990 bp**	**12**	**26**	**39**	**28**						**+++**			**yes**
PB000977.02.0	**LCCL adhesive-like protein (PbSLAP, PbSR) (female)**	**1487 bp**	**10**	**26**		**24**						**+++**			**yes**
PB001094.00.0	**NIMA-related kinase NEK4, putative (female)**	**678 bp**	**7**	**11**	**10**	**8**						**++**			**yes**
PB000791.03.0	**hypothetical protein (male)**	**1527 bp**					**42**	**11**	**45**	**60**			**+++**		**yes**
PB000733.03.0	**hypothetical protein (male)**	**1576 bp**					**25**		**27**	**32**			**++**		**yes**
PB000198.00.0	conserved hypothetical protein	758 bp	18	24		20	15	18	17	20		+++	+++		yes
PB000245.01.0	proteasome regulatory subunit, putative	1457 bp	5	6	8	3		5		4	8	++	++	++	yes
PB300655.00.0	conserved hypothetical protein	704 bp		10			4	10		14	9	+	++	+	yes
PB000530.02.0	hypothetical protein S/T kinase	886 bp		6		6	5	3	4	6	3	+	+++		yes/?[a]
PB000119.00.0	transcriptional regulator, putative	646 bp					24		25	27	9	+	+++		?[b]

Promoter regions (bp, size in base pairs) of genes were selected on basis of the proteome data. Numbers given in this table are for unique peptides (i.e., not sharing the same sequence), which correspond to the given protein. GFP expression was assessed by fluorescence microscope examination of asexual blood stages and activated male gametocytes (exflagellating) and female gametocytes (emerged from the host erythrocyte). The relative GFP fluorescence intensity of the different stages is scored with a "+" sign. The promoters of three female-specific and two male-specific proteins that have a sum total of three or more unique peptides have been highlighted (bold). The highly purified samples of male and female gametocytes are indicated by bold and underlined text.

[a] Fluorescence was not observed in asexual-stage parasites as predicted but was observed in young gametocytes.

[b] The predicted fluorescence in asexual parasites was not observed, but female gametocytes show a low level of fluorescence.

Gender-Specific Distribution of Protein Kinases Reflects Function

Within the predicted *Plasmodium* proteomes, conserved protein kinases (PKs) and their counterparts, phosphatases, constitute a significant proportion of the total predicted enzymes (~100 genes). PKs are in general responsible for signaling within and between cells and effect transitions from one cellular state to the next. *Plasmodium* is thought to lack tyrosine or STE kinases (Ward et al., 2004). Our *P. berghei* proteome analyses confirmed this and revealed 60 PKs and 16 protein phosphatases, of which a large proportion were gametocyte specific (ten kinases and ten phosphatases; Table 3). These enzymes could be further divided, as two were identified as female specific and six as male specific. The role of kinases in sexual development is known for only one protein, CDPK4 (calcium-dependent protein kinase 4), which has essential roles in the formation of the male gamete and the maturation of the fertilized zygote (Billker et al., 2004) and is found in both the MG (71 peptides) and FG (16 peptides) proteomes (Table 3).

To confirm their sex specificity and investigate their role in sexual development, we selected two PKs for further analysis by targeted disruption of their genes: a female-specific putative NIMA-related kinase, NEK4 (PB001094.00.0; *P. falciparum* ortholog: MAL7P1.100) and a male-specific putative mitogen-activated protein (MAP) kinase, MAP2 (PB000659.00.0; *P. falciparum* ortholog: PF11_0147). NIMA-related kinases are implicated in a variety of cell cycle control events, including mitosis and meiosis, and four *Plasmodium* homologs have been identified (O'Connell et al., 2003; Ward et al., 2004). The MAP kinases are commonly involved in proliferation and differentiation of cells, among many other roles (Lewis et al., 1998), and two MAP kinases have been identified in *Plasmodium* (Doerig et al., 1996; Dorin et al., 1999).

In four independent experiments using standard methodologies involving double-crossover gene replacement, we obtained and cloned two *P. berghei* lines deficient in NEK4 and two lines deficient in MAP2 (i.e., NEK4$^-$ and MAP2$^-$; Figure 3A). Correct disruption of the genes in these lines was shown by standard PCR analysis and by the absence of transcripts through Northern and RT-PCR analysis (Figure 3B). The MAP2$^-$ parasites produced FG and MG in wild-type numbers with normal light-microscope morphology, but male gamete formation was completely inhibited. Gametogenesis could be induced, resulting in the escape of the male gamete from the host cell, followed by genome replication. Interestingly, further development was arrested and neither nuclear division nor gamete formation occurred, resulting in the presence of rounded male gametes with a single, enlarged nucleus (Figure 3B) containing multiple copies of the genome (data not shown). However, female gametes could develop completely normally, as was shown by the formation of ookinetes in crossfertilization with fertile males of the mutant line P47$^-$ of *P. berghei* that produce infertile females (Figure 3C). The observed effect of disruption of *map2* on male gametes is consistent with the exclusive identification of the MAP protein within the MG proteome. The specific effect of the absence of MAP2 on the process of nuclear division is consistent with the role of MAP kinases in cell proliferation and further

Table 2. Distribution of Unique Peptides of Proteins Predicted to Be Associated with the Axoneme/Flagella of the Male Gamete

				Female				Male				
Accession Number	Protein Name	*P. falciparum* Gene Model	Mixed	1	2	3	4	1	2	3	4	Asex
PB001056.02.0	dynein heavy chain, putative	PF10_0224	32					35	9	47	45	
PB000791.03.0	putative heavy chain dynein—phosphatase	PFI0260c	23					42	11	45	60	
PB000989.00.0	**dynein heavy chain, putative**	**MAL7P1.162**	**14**	**14**	**27**		**41**					
PB001397.02.0	hypothetical protein kinesin, putative	PFA0535c	10					9		7	16	
PB000781.01.0	PF16 protein, putative	PF11_0318	7		3			8		5	11	
PB000733.03.0	hypothetical protein dynein heavy chain, putative	PF11_0240	7					25		27	32	
PB000182.03.0	calmodulin, putative ODA-DC	PF14_0323	5					2			9	1
PB000287.00.0	kinesin-like protein, putative	MAL12P1.431	4		1	1		4		3	2	
PB000535.01.0	outer-arm dynein light chain 2, putative	MAL8P1.46	4					4	6		8	
PB000059.02.0	possible PR2 homolog *C. reinhardtii* axoneme	PF11_0433	4					3		2	4	
PB001662.02.0	dynein-associated protein, putative	PF14_0243	3					5		5	8	
PB001391.02.0	centrin, putative Ca binding EF hand	PFA0345w	3							3	3	
PB300889.00.0	homolog of DC3 outer dynein arm—docking-complex protein from *C. reinhardtii*	PF14_0420	1					1	1		1	
PB000808.00.0	dynein-associated protein, putative	PF14_0202	1							1	1	
PB001200.02.0	dynein light chain 1, putative	MAL12P1.132	1							1	5	
PB000005.01.0	hypothetical dynein heavy chain C-term	PF14_0626						10		2	9	
PB000607.03.0	conserved hypothetical protein—radial spoke protein 6 homolog *C. reinhardtii*	PF11_0057						3		6	1	
PB000269.01.0	chromosome segregation protein, putative	MAL13P1.96						3			7	
PB001113.01.0	conserved WD40 domain homology to *C. reinhardtii* IC dynein protein IC138	PFI1080w						2		7		
PB000354.00.0	dynein light chain type 2, putative	PF11_0148						2		1	2	
PB000316.00.0	putative ODA1 homolog, ODA-DC protein	MAL8P1.12						1				
PB000659.03.0	tctex1 homolog *C. reinhardtii* dynein light chain regulator—binds PK	PFI1350c						1			2	
PB100371.00.0	hypothetical protein—possible radial-spoke protein axoneme	PF14_0030						1			1	
PB001018.01.0	dynein 14 kDa light chain, flagellar outer arm, putative	PF14_0590								1		

Proteins predicted to be associated with the axoneme/flagella are selected based on published data (for references see text). PB000989.00.0 is highlighted in bold, as it is the only dynein that is not found in the male proteome but is found exclusively in the female gametocyte. The highly purified samples of male and female gametocytes are indicated by the bold and underlined text.

defines the kinase cascade and the cellular events in male-gamete activation (see Discussion).

In contrast to the MAP2$^-$ parasites, the NEK4$^-$ parasites produced both male and female gametes in wild-type numbers with normal morphology by light microscopy. Moreover, fertilization did occur at wild-type levels, as was shown by the presence of zygotes (Figure 3B). However, further development was severely affected, resulting in complete absence of mature ookinetes, and only arrested zygotes were present at 16–18 hr after fertilization (Figure 3B). Crossfertilization studies with fertile female gametes from an independent mutant parasite line P48/45$^-$ that produce defective males demonstrated that NEK4$^-$ male gametes were able to fertilize female gametes that developed in mature ookinetes at wild-type levels (Figure 3C). Interestingly, females of NEK4$^-$ fertilized by fertile males of P47$^-$ were similarly not able to overcome the arrest of zygote development, demonstrating that the simple presence of a complete *nek4* gene obtained from the male cannot complement the female defect that confirms the gender specificity of NEK4 expression (Figure 3C).

The influence of NEK4 on the cell cycle of the zygote was further investigated by a flow cytometric analysis of the DNA content of arrested NEK4$^-$ zygotes 2–3 hr after fertilization (Figure 4). In wt zygotes, DNA replication up to the tetraploid DNA content coincides with meiotic division at 2–3 hr after fertilization (Janse et al., 1986a; Sinden, 1991). The arrested NEK4$^-$ zygotes show a clear diploid DNA content without the meiotic DNA replication after fusion of the female and male nucleus (Figure 4B). This observation indicates that NEK4 is important either just before or during meiosis, and this specific role during zygote formation is in agreement with the exclusive detection of NEK4 in the FG proteome.

Table 3. Distribution of Unique Peptides of Protein Kinases and Phosphatases that Were Exclusively Detected in Gametocytes

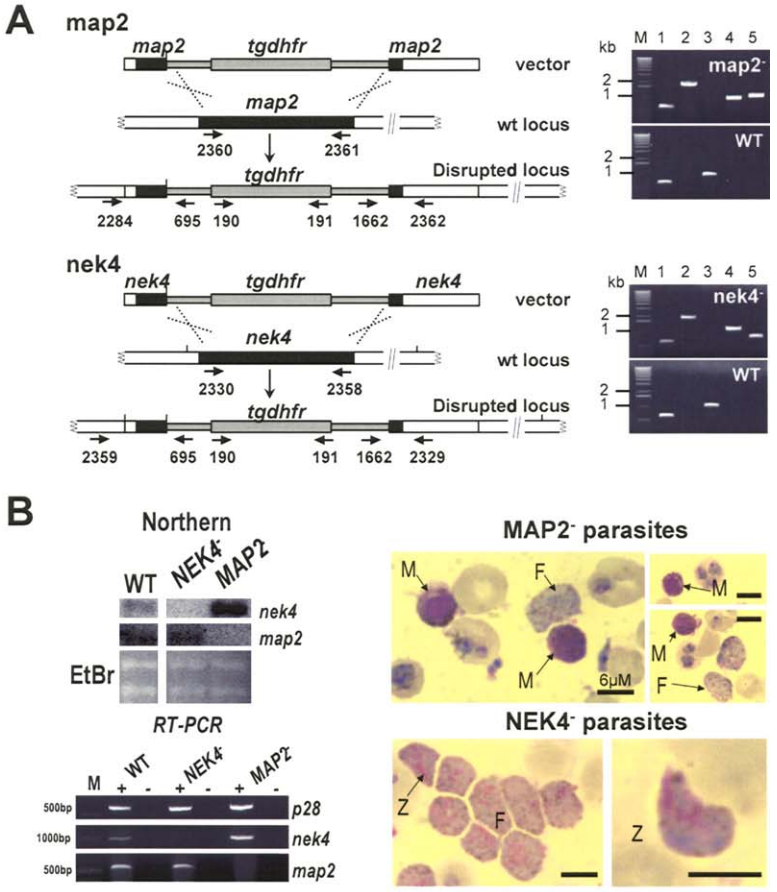
P. berghei Accession Number	Protein Name	*P. falciparum* Gene Model	% Sim	Mixed	Female 1	Female 2	Female 3	Female 4	Male 1	Male 2	Male 3	Male 4	Asexual
Gametocyte Specific													
PB000659.00.0	**mitogen-activated protein kinase MAP2, putative (male)**	**PF11_0147**	**84**	**3**					**7**	**4**	**4**	**8**	
PB001392.02.0	**NIMA-related kinase NEK1 (male)**	**PFL1370w**	**79**						**4**			**1**	
PB000031.01.0	protein phosphatase, putative	PF08_0129	90	2		2			3		3	2	
PB000558.02.0	**low-confidence phosphatases (male)**	**PFL0445w**	**78**	**5**					**3**		**1**	**5**	
PB000597.03.0	serine/threonine protein phosphatase PbPp5	MAL13P1.274	79		2	5			3			7	
PB001088.00.0	**protein phosphatase 2b regulatory subunit, putative (male)**	**PF14_0492**	**96**						**2**		**1**	**2**	
PB000945.02.0	**hypothetical protein phosphatase 2C (male)**	**MAL8P1.109**	**81**						**1**	**1**		**2**	
PB000254.01.0	protein kinase, putative	MAL12P1.490	88		1				1			2	
PB001001.01.0	casein kinase II, alpha subunit, putative	PF11_0096	98	11		4		3	1			5	
PB001179.01.0	cdc2-like protein kinase, putative	MAL6P1.271	82	2					1				
PB000365.01.0	phosphatase 1 regulatory subunit, putative	PFE0455w	79						1				
PB001204.00.0	phosphotyrosyl phosphatase activator, putative	PF14_0280	87					1	1			1	
PB000293.00.0	protein kinase, putative	MAL13P1.279	95			1					2	1	
PB000189.00.0	**serine/threonine protein phosphatase, putative (male)**	**PFC0595c**	**97**								**2**	**2**	
PB001094.00.0	**NIMA-related kinase NEK4, putative (female)**	**MAL7P1.100**	**98**	**10**	**7**	**11**	**10**	**8**					
PB001201.00.0	putative phosphatase	PF14_0282	70		1								
PB000670.02.0	**casein kinase II beta chain, putative (female)**	**PF11_0048**	**91**	**3**		**3**		**4**					
PB000606.01.0	protein phosphatase, putative	MAL8P1.108	90			1							
PB000491.01.0	hypothetical protein Ser/Thr protein kinase, putative	PF14_0408	70	1									
PB000567.00.0	hypothetical protein pyridoxine kinase	MAL6P1.266	80					2					
PB000600.00.0	**calcium-dependent protein kinase, putative CDPK4 (shared)**	**PF07_0072**	**97**	**22**	**4**	**8**	**4**		**12**	**22**	**16**	**21**	**5**

Proteins predicted to be protein kinases and phosphatases were selected based on protein predictions in GeneDB and PlasmoDB (see text for URLs). Only those kinases and phosphatases that were detected in the gametocyte proteomes and not in the asexual blood stages are shown. Two female-specific and six male-specific protein kinases/phosphatases that have a sum total of three or more unique peptides have been highlighted (bold). In addition, the calcium-dependent protein kinase 4 (CDPK4) that is involved in male gamete regulation but found in both genders is highlighted (bold). The highly purified samples of male and female gametocytes are indicated by bold and underlined text.

Discussion

Postgenome surveys have revealed the extensive novel gene and protein expression that accompanies the development of *Plasmodium* gametocytes. However, to date, little distinction has been made between proteins and processes that are specific to either MG or FG because it has not been possible to separate and purify the sexes. The generation of two lines of *P. berghei* expressing *gfp* under the control of different promoters has here permitted the first separation of MG from their FG counterparts using flow cytometry. Subsequent analysis of purified MG and FG by LC/MS-MS generated extensive gender-specific proteome lists that were validated by the creation and analysis of individual transgenic parasite lines that expressed *gfp* under control of the promoter from ten different candidate genes. Comparison of the asexual subtracted MG and FG proteomes confirmed that MG and FG are highly specialized cell forms yet are extensively diverged from one another. In our analysis they shared only 69 gametocyte-specific proteins, reflecting both their different roles and their readiness for subsequent development after activation in the mosquito midgut. The MG protein complement of 650 proteins, of which 36% (236) are unique to the male (relative to asexual blood stages), reveals it as the most distinct life-cycle stage so far analyzed in *Plasmodium*. In fact, 17% of this MG proteome is also unique compared to all other *Plasmodium* proteomes analyzed, including the other gametocyte and gamete proteomes. One hundred and one proteins were designated as FG specific. In our analysis we identified 934 proteins in the gametocyte stages, and this corresponded to either 79% of the 484 proteins previously identified in *P. berghei* (Hall et al., 2005) or 52% of the 834 orthologs cumulatively identified in two studies in *P. falciparum* (Florens et al., 2002; Lasonder et al., 2002).

The exact mechanisms of regulation of gene expression in *Plasmodium* are poorly understood (reviewed in Horrocks et al., 1998). Gene structure appears to be typically eukaryotic, with promoter activity generally upstream of transcriptional start sites and relying upon certain ill-defined elements that show little intra- or interspecies conservation. Our GFP promoter-tagging studies demonstrate that expression of sex-specific proteins can be controlled at the transcriptional level by either individual gene promoters of transcription or increased stability of specific mRNA populations mediated by the 5′UTR. We performed an initial analysis using the motif-identifier program MEME (Bailey and Gribskov, 1998) to identify putative motifs present in the



C

Cross	Exflagellation	Ookinete conversion rate (SD)	Gamete fertility
WT	wt	62.3 (3.51)	M+F+
P47-	wt	0	M+ F-
P48/45-	wt	0	M- F+
NEK4-	wt	0	?/?
MAP2-	0	0	M-/?
P47- x P48/45-	-	65.3 (4.11)	M+ x F+
P47- x NEK4-	-	0	M+ x F-
P47- x MAP2-	-	57.3 (5.03)	M+ x F+
P48/45- x NEK4-	-	60.7 (6.6)	F+ x M+
P48/45- x MAP2-	-	0	F+ x M-
NEK4- x MAP2-	-	62.7 (6.6)	M+ x F+

Figure 3. Generation and Analysis of Two Parasites Lines, MAP2$^-$ and NEK4$^-$, that Are Deficient in the Protein Kinases MAP2 and NEK4

(A) Schematic representation of the replacement vectors used to disrupt *map2* and *nek4* and the genomic loci before and after insertion of the vectors. Both vectors contain the *tgdhfr-ts* selection cassette. The location of primers (arrows) that have been used to show correct disruption of the loci (see [B]) are shown. Open boxes, untranslated regions; black boxes, coding regions; gray box, *tgdhf-ts* selection cassette. Correct disruption of both *map2* and *nek4* as shown by PCR (right-hand panels). Lane 1: amplification of *p28* of *P. berghei* using primers 537 and 538 as a control PCR. Lane 2: amplification of the *tgdhf-ts* with primers 190 and 191. Lane 3: amplification of the ORF, i.e., *map2* (primers 2360/2361) and *nek4* (primers 2330/2358). Lane 4: confirmation of 5′ integration of the vector, i.e., *map2* (primers 2284/695) and *nek4* (primers 2359/695). Lane 5: confirmation of 3′ integration of the vector, i.e., *map2* (primers 1662/2362) and *nek4* (primers 1662/2329). For the expected sizes of the PCR fragments, see Experimental Procedures, and for the sequence of the primers, see Table S2.

(B) Northern analysis and RT-PCR using mRNA of purified gametocytes showed an absence of transcription of *map2*$^-$ and *nek4*$^-$ in the mutant lines. Northern analysis was performed on the mRNA extracted from wild-type (WT), NEK4$^-$, and MAP2$^-$ gametocytes hybridized to either a *nek4* locus- (primers 2330/2372) or *map2* locus- (2360/2361) specific probe. The ethidium bromide (EtBr) staining of ribosomal RNA shows the relative amount of RNA loaded per lane. RT-PCR was performed on the same RNA in the presence (+) or absence (–) of reverse transcriptase, and the following transcripts were amplified: *p28* (a gametocyte-specific gene as a positive control; 537/538), *nek4* (2412/2329), and *map2* (2360/2414). For the expected sizes of the PCR fragments, see Experimental Procedures, and for the sequence of the primers, see Table S2.

Phenotype of the MAP2$^-$ and NEK4$^-$ parasites (right-hand panels) as shown by light-microscopic morphology of the gametocytes/gametes/zygotes in ookinete culture 16–18 hr after activation of gamete production. In MAP2$^-$, no exflagellation and ookinete formation is observed, and all MG (M) appear as rounded cells, escaped from the host erythrocyte, with a central, enlarged dark-stained nucleus in which genome replication has taken place. In NEK4$^-$, exflagellation is normal but no ookinetes are formed. Cultures contain clusters of both unfertilized female gametes (single nucleus) and aborted zygotes (Z), cells with enlarged nuclei compared to the unfertilized gametes and with the characteristic protrusions of early ookinete development (Janse et al., 1985). All scale bars (in black) represent 6 μM.

(C) Fertility of MG and FG gametes of MAP2$^-$ and NEK4$^-$ parasites tested in crossfertilization assays. Fertility is determined by the ability of gametes of MAP2$^-$ and NEK4$^-$ to develop into ookinetes after crossfertilization with mutant lines that are defective in the production of either male (P48/45$^-$) or female gametes (P47$^-$). The ookinete conversion rate is the percentage of female gametocytes/gametes that develop into mature ookinetes, 16–18 hr after activation of gamete production. wt, wild-type parasites of cl15cy1. SD, standard deviation.

5′UTR of male- and female-specific genes that might be involved in sex-specific expression, but so far, the results of these analyses are inconclusive (data not shown).

The FG and MG proteomes confirm the anticipated gender-specific distribution of the observed morphologically distinctive features of the male and female gametocyte. These proteomes provide much greater mo-

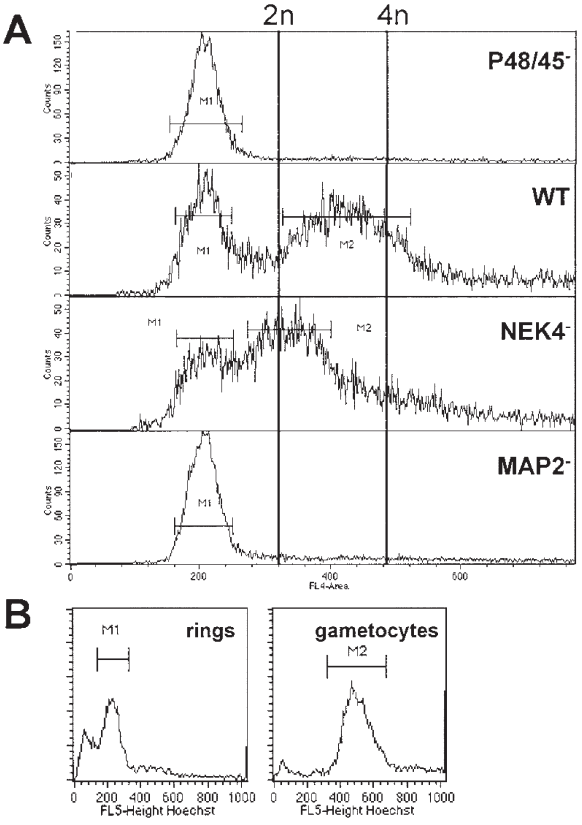


Figure 4. DNA Content of Female Gametes and Zygotes of MAP2⁻ and NEK4⁻ Parasites, 2–3 Hours after Activation of Gamete Production of Purified Gametocytes

(A) The DNA content is defined as the fluorescence intensity of cells as measured by flow cytometry after staining with the DNA-specific dye Hoechst 33258. Female gametes and zygotes were selected using size measurements by gating on forward/sideward scatter as shown in Figures 1D and 1E. The male gamete defective 48/45⁻ parasites are used as a control for the DNA content of unfertilized female gametes (first peak in upper and lower panel; see also [B]). The wt parasites of cl15cy1 are used as a control for the DNA content of zygotes that have been fertilized (second, broad peak in second panel; the first peak represents the unfertilized female gametes), showing a DNA content between 3 and 4 times the haploid value (3–4N) as a result of meiotic DNA replication (Janse et al., 1986b). In the NEK4⁻ parasites, unfertilized gametes (first peak) and fertilized gametes (second peak) showing a diploid DNA content (2N) are present.

(B) The fluorescence intensity of the haploid DNA content (1N) has been defined by the fluorescence intensity of the haploid ring stages (left panel) (Janse et al., 1987). Female gametocytes and unfertilized female gametes show a fluorescence intensity that is between 2 and 2.3 times the haploid DNA content (right panel) as reported before (Janse and van Vianen, 1994). Based on these values, the 2N and 4N values have been calculated for as shown in (A).

lecular detail to support these structural studies but also clarify the recent transcriptome/proteome analyses of mixed male and female gametocytes, ultimately revealing some unexpected protein distributions. Thus, the expected bias of the MG proteome to proteins associated with both genome replication and axoneme formation was observed. We identified 26 proteins associated with flagellar complex within the annotated genome of *Plasmodium berghei*, of which 22 were sequenced in our analysis (Inaba, 2003; Ostrowski et al., 2002). Twenty-one of these twenty-two were uniquely expressed in the gametocyte stages, 19 being specific to the MG proteome. Unexpectedly, one predicted cytoplasmic dynein protein (PB000989.00.0) was FG specific; this will be investigated in the context of the role of cytoplasmic dyneins with organelle transport (Mallik and Gross, 2004). The distribution of proteins of the mitochondrion and protein synthetic machinery were strongly FG biased. Interestingly, apicoplast proteins are as abundant in males as in females, while it is known that only maternal transfer of the apicoplast genome occurs (Creasey et al., 1994; Vaidya et al., 1993). Thus, the apicoplast most likely also has a function during the development of the male gametocyte (see Table S6).

Both the mature FG and MG are in a state of cell cycle arrest in the blood, and re-entry of the cell cycle is triggered by various environmental stimuli initiating rapid and extensive cellular responses. It has been shown that gametocyte activation to form gametes is initiated by changes in environmental temperature, pH, and xanthurenic acid (XA) (Billker et al., 1997; Billker et al., 1998). Although the receptor for XA has yet to be identified, it is clear that activation of gamete formation and subsequent events are greatly influenced by intracellular calcium homeostasis operating at least in part through a CDPK4-controlled signal transduction cascade (Billker et al., 2004). Male gametocytes of mutant *P. berghei* parasites deficient in CDPK4 failed to replicate their genome and produce gametes, and CDPK4 was present in the proteomes of asexual blood stages, MG, and FG. The presence of protein kinases and phosphatases that are only expressed in either males or females provides strong evidence that there are sex-specific refinements of signal transduction. Indeed, targeted disruption of two genes encoding protein kinases confirmed that *Plasmodium* gametocytes maintain gender-specific signaling pathways. MG of mutants that lack the male-specific MAP2 kinase re-enter the cell cycle after activation, complete genome replication, but fail to enter into nuclear division. MAP kinases are serine/threonine protein kinases commonly involved in the proliferation of cells (Lewis et al., 1998). Two MAP kinases have been identified in the *Plasmodium* genome, MAP1 and MAP2: MAP1 appears to have a classical Thr-X-Tyr activation site, whereas MAP2 appears to have a diverged Thr-Ser-His site (Dorin et al., 1999). Unusually, no obvious orthologs of upstream kinases (either MAP-kinase-kinase or MAP-kinase-kinase-kinase) have been annotated in any genome of *Plasmodium*, suggesting alternative activation cascades (Dorin et al., 2005; Ward et al., 2004). However, it has been observed that NEK1 of *P. falciparum* can phosphorylate MAP2 in vitro, indicating that NEK1 may be acting as a MAP-kinase-kinase (Dorin et al., 2001). Interestingly, in our analysis, NEK1 (PB001392.02.0) is indeed one of the five remaining MG-specific kinases. The presence and significance of NEK1 and, in particular, MAP2 along with the known upstream response of CDPK4 to extracellular signals will help to decipher the sequence of molecular events necessary in male gamete activation and formation.

In contrast to MAP2, the FG-specific NIMA-related kinase, NEK4, appears not to be involved in gamete

formation but plays a vital role during further development of the zygote and progression into meiosis. Indeed, the gametes of the NEK4$^-$ parasites are able to fertilize, but the zygotes are arrested at the diploid stage of development. NIMA kinases are also serine/threonine kinases, which are generally involved in controlling and coordinating signaling processes in mitosis (O'Connell et al., 2003). The NEK proteins, first characterized in the filamentous fungus, *Aspergillus nidulans*, are implicated in a variety of cell cycle control events (Lu and Hunter, 1995). However, very little is known about NEK4, which is likely to be the ortholog of human STK2, where, interestingly, expression is observed in meiotic tissue, namely the testis (Chen et al., 1999).

Only 10–30 min elapse between uptake of gametocytes by a mosquito, gamete formation, and fertilization, encompassing many rapid changes at the cellular level in an environment completely different from the blood circulation in which the gametocytes developed. Rapidity of these cellular events may explain why both MG and FG undertake storage of proteins that, as our data clearly show, are only exploited upon receipt of the appropriate extracellular signals, e.g., proteins of the axoneme and protein kinases are clearly stockpiled. In addition to storage of proteins, it has been shown that mRNA species encoding proteins, which are required for development subsequent to fertilization (e.g., P28), are stored in the female gametocyte as repressed mRNA species (Hall et al., 2005). Accordingly, both these stored, inactive proteins and repressed mRNA transcripts may be activated in an ordered progression upon activation of gamete formation, a process likely to be regulated by phosphorylation. This also raises the interesting question of why the female gametocyte makes a distinction in the storage of molecules as either proteins or repressed mRNA, which will require further investigation.

This study not only provides the most comprehensive list of gametocyte-specific proteins and the first list of gender-specific proteins presently available in *Plasmodium* but, importantly, also allows for identification of proteins that control the multiple cellular functions involved in the transmission of the parasite from the vertebrate host to the mosquito. Our analysis has assigned a number of proteins to previously characterized features and functions of *Plasmodium* gametocytes and gametes. We showed that some of these proteins, which are necessary for the subsequent stages of development, are presynthesized and stored, ready for rapid deployment in the mosquito midgut, which is an environment extremely different from the mammalian blood-circulatory system. Further investigations informed by the MG and FG proteomes should provide new insights into both the biology of parasite sex and the strategies the parasite uses for survival in the mosquito midgut. This in turn may lead to the identification of targets for the interruption of transmission, either by drugs or vaccines.

Experimental Procedures

Construction of the pPbGFP$_{CON}$ and pPbGFP$_{TUB}$ Vectors

The construction of the pPbGFP$_{CON}$ vector for expression of GFP under the *ef1aa* promoter has been described (Franke-Fayard et al., 2004) and formed the basis for the generation of vector PbGFP$_{TUB}$, in which the *gfp* is placed under the control of the *α-tubulin-II* promoter. The promoter region of PbGFP$_{CON}$ was replaced by a 1.3 kb promoter fragment (amplified using primers L1516 and L1517) of the *P. berghei α-tubulin-II* gene (Rawlings et al., 1992). The *d-ssu-rrna* fragment integrates into the genome by single-crossover homologous recombination into either the *c-* or *d-rrna* gene unit (Franke-Fayard et al., 2004) after linearization of the pPbGFP$_{TUB}$ vector at the unique ApaI and KspI sites. For primer sequences see Table S2.

Parasites, Transfection, and Analysis of PbGFP$_{CON}$ and PbGFP$_{TUB}$ Parasites

The reference clone (clone 15cy1; wild-type parasites) of the ANKA strain of *P. berghei* was used to generate both PbGFP$_{TUB}$ and the previously characterized PbGFP$_{CON}$ (Franke-Fayard et al., 2004) parasites. Transfection of *P. berghei*, selection of PbGFP$_{TUB}$ parasites, and their characterization were as described for PbGFP$_{CON}$ (Franke-Fayard et al., 2004). GFP fluorescence was visualized using fluorescence MDR microscopy (Leica; GFP filter settings) and images recorded using a DC500 digital camera.

A nongametocyte-producer clone (HPEclone1; Janse et al., 1989) of the ANKA strain of *P. berghei* was used for asexual blood-stage proteome analysis. Mixed asexual stages, including trophozoites and immature and mature schizonts (10^8 cells), were generated for subsequent analysis (Janse and Waters, 1995).

Mixed FG and MG gametocytes for protein analysis were obtained using the method of Beetsma et al. (1998), and 8×10^7 to 4×10^8 cells, consisting of more then 90% mature gametocytes with less than 10% (degenerated) asexual blood-stage contamination, were obtained.

Purification of Gametocytes and Separation of Males and Females by Flow Cytometry

Purified populations of mixed FG and MG of PbGFP$_{CON}$ and PbGFP$_{TUB}$ parasites (8×10^7 to 4×10^8 cells) were used to separate the FG and MG by flow cytometry cell sorting. Just before sorting, the purified gametocytes were rapidly cooled down to 4°C in 3 ml enriched PBS (20 mM HEPES, 20 mM glucose, 4 mM $NaHCO_3$, 0.1% BSA [pH 7.25–7.30]) and sorted at 4°C using a FACS Vantage SE (Becton Dickinson, California). Excitation of cells was at 488 nm, and GFP emission was detected using a band-pass filter of 530/30 nm. Gametocyte-infected RBC were selected by gating on forward/side-light scatter, and FG and MG were sorted on basis of GFP fluorescence by setting gates as shown in Figure 1. Gametocytes were collected over a 7 hr period, and in each sorting, between 3 and 4.2×10^7 parasites were collected. Sorted gametocytes were collected in enriched PBS, pelleted by centrifugation (2000 rpm), and "snap" frozen in liquid nitrogen. Sorted gametocytes were able to form gametes after activation in vitro (data not shown).

LC-MS/MS Analysis of Malaria Proteins

All subsequent analyses were performed on 1×10^7 parasites. Protein mixtures of all samples were extracted in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and separated into ten fractions by electrophoresis on a 10% PAGE gel. Proteins were treated with dithiothreitol (DTT) and iodoacetamide and in-gel digested by trypsin, and these peptides were analyzed by LC-MS/MS as described (Lasonder et al., 2002). Essentially, peptide mixtures were loaded onto 100 μm ID columns, packed with 3 μm C18 particles (Vydac), and electrosprayed into a quadruple time-of-flight mass spectrometer (QSTAR, Sciex-Applied Biosystems). Fragment ion spectra were recorded using information-dependent acquisition and duty-cycle enhancement. To acquire MS/MS spectra of the peptides, samples were measured at least three times with exclusion lists (Lasonder et al., 2002). In total, more than 300 LC-MS/MS runs were acquired, resulting in more than 50,000 MS/MS spectra per sample. Malaria proteins were identified by searching combined protein databases of *P. berghei*, *Mus musculus*, and human IPI using Mascot (Matrix Science). Identification criteria were as in Lasonder et al. (2002) (see Supplemental Data).

Promoter Analysis by GFP Tagging

Ten genes were selected (see Table 1) to investigate the role of the 5′UTRs (promoter regions) in controlling sex-specific gene expres-

sion. These regions of these genes were PCR amplified (primers shown in Table S2) and cloned in vector $pPbGFP_{CON}$ as described above. Transfection and selection of parasites expressing GFP was as described above. GFP expression was monitored in parasites obtained from asynchronous blood-stage infections containing rings, trophozoites, young schizonts, and gametocytes. MG- and FG-specific expression was determined in gametocytes 10–15 min after in vitro stimulation of gamete formation (Janse and Waters, 1995), allowing discrimination between the exflagellating male gametocyte and the female gametocyte emerging from the erythrocyte.

Generation and Genotype Analysis of MAP2$^-$ and NEK4$^-$ Parasite Lines

To disrupt *map2* and *nek4*, replacement vectors were made using vector pb3D transformed into *P. berghei*, selected, cloned, and verified using standard methodologies (Franke-Fayard et al., 2004) (see Supplemental Data for details).

Phenotype Analysis of MAP2$^-$ and NEK4$^-$ Parasites

Gametocyte and gamete production and subsequent fertilization and zygote development were determined in a previously developed in vitro fertilization assay (Janse and Waters, 1995; van Dijk et al., 2001). The conversion of female gametocytes into ookinetes (i.e., fertilization rate) is defined as the percentage of female gametocytes or gametes that develop into mature ookinetes. Both MAP2$^-$ and NEK4$^-$ parasites did not produce mature ookinetes after self-fertilization. To distinguish between male and female defects, we tested the fertility of male and female gametes in crossfertilization assays (van Dijk et al., 2001) by crossfertilization of the MAP2$^-$ and NEK4$^-$ gametes with different mutant *P. berghei* lines that have either defective male gametes (P48/45$^-$; van Dijk et al., 2001) or defective female gametes (P47$^-$; M.R. van Dijk, personal communication). Eighteen hours after crossing the gametes, the number of unfertilized female gametes and ookinetes were counted and the conversion rate of female gametocytes/gametes into ookinetes determined as described above.

The DNA content of gametocytes/gametes and zygotes was determined by FACS analysis of parasites stained with the DNA-specific dye Hoechst 33258 (modified from Janse et al., 1987; Janse and van Vianen, 1994). Purified gametocyte populations were generated, as described above, from the following parasite lines: wild-type parasites (cl15cy1), mutant P48/45$^-$ parasites that produce infertile male gametes and fertile female gametes (van Dijk et al., 2001), MAP2$^-$, and NEK4$^-$. Gametocytes (3×10^6) were suspended in 1 ml of "ookinete culture medium" at 21°C, as described (van Dijk et al., 2001), to activate gamete production. These cell suspensions were kept at 21°C for a period of 2.5 hr for fertilization and subsequent DNA replication in zygotes to occur (Janse et al., 1986b). Live cells were then stained with Hoechst 33528 (10 μM) for 1 hr at 21°C. All experiments were performed in triplicate. Fluorescence intensity of the cells was measured on a FACS Vantage SE (Becton Dickinson) after UV excitation using a high-power argon-ion laser (350 nm). Cells (unfertilized female gametes, zygotes) were selected on forward/sideward light scatter, and the blue Hoechst fluorescence was detected using 424/24 filters. The fluorescence intensity of 50,000 cells per sample was measured, and data analysis was performed using the Cellquest software (Becton Dickinson). As a reference for the fluorescence intensity of haploid (1N) DNA content of the parasites, we used Hoechst-stained ring-infected blood stages as described (Janse et al., 1987).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and eight tables and are available with this article online at http://www.cell.com/cgi/content/full/121/5/675/DC1/.

Acknowledgments

The authors wish to thank G. McFadden and G. van Dooren for annotated lists of *Plasmodium* mitochondrial and apicoplast proteins. M. van Dijk is thanked for providing parasite clones in advance of publication. We would also like to thank R. van der Linden and M. van der Keur for all their help and expertise in using the flow cytometry equipment and the CEBI group for all their assistance and helpful discussions. In particular, we would like to gratefully acknowledge J. Ramesar for all his technical support. This work was supported by the Dutch Science Foundation (NWO/Genomics grant number 050-10-053), the Wellcome Trust Functional Genomics Initiative, Leiden University Medical Centre, Stimuleerings Fonds, and the Danish National Research Foundation.

Received: February 2, 2005
Revised: March 10, 2005
Accepted: March 22, 2005
Published: June 2, 2005

References

AnamikaSrinivasan, N., and Krupa, A. (2005). A genomic perspective of protein kinases in Plasmodium falciparum. Proteins *58*, 180–189.

Bailey, T.L., and Gribskov, M. (1998). Methods and statistics for combining motif match scores. J. Comput. Biol. *5*, 211–221.

Beetsma, A.L., van de Wiel, T.J., Sauerwein, R.W., and Eling, W.M. (1998). Plasmodium berghei ANKA: purification of large numbers of infectious gametocytes. Exp. Parasitol. *88*, 69–72.

Billker, O., Shaw, M.K., Margos, G., and Sinden, R.E. (1997). The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of Plasmodium berghei in vitro. Parasitology *115*, 1–7.

Billker, O., Lindo, V., Panico, M., Etienne, A.E., Paxton, T., Dell, A., Rogers, M., Sinden, R.E., and Morris, H.R. (1998). Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. Nature *392*, 289–292.

Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004). Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. Cell *117*, 503–514.

Carter, R. (2001). Transmission blocking malaria vaccines. Vaccine *19*, 2309–2314.

Chen, A., Yanai, A., Arama, E., Kilfin, G., and Motro, B. (1999). NIMA-related kinases: isolation and characterization of murine nek3 and nek4 cDNAs, and chromosomal localization of nek1, nek2 and nek3. Gene *234*, 127–137.

Creasey, A., Mendis, K., Carlton, J., Williamson, D., Wilson, I., and Carter, R. (1994). Maternal inheritance of extrachromosomal DNA in malaria parasites. Mol. Biochem. Parasitol. *65*, 95–98.

Doerig, C.M., Parzy, D., Langsley, G., Horrocks, P., Carter, R., and Doerig, C.D. (1996). A MAP kinase homologue from the human malaria parasite, Plasmodium falciparum. Gene *177*, 1–6.

Dorin, D., Alano, P., Boccaccio, I., Ciceron, L., Doerig, C., Sulpice, R., Parzy, D., and Doerig, C. (1999). An atypical mitogen-activated protein kinase (MAPK) homologue expressed in gametocytes of the human malaria parasite Plasmodium falciparum. Identification of a MAPK signature. J. Biol. Chem. *274*, 29912–29920.

Dorin, D., Le Roch, K., Sallicandro, P., Alano, P., Parzy, D., Poullet, P., Meijer, L., and Doerig, C. (2001). Pfnek-1, a NIMA-related kinase from the human malaria parasite Plasmodium falciparum Biochemical properties and possible involvement in MAPK regulation. Eur. J. Biochem. *268*, 2600–2608.

Dorin, D., Semblat, J.P., Poullet, P., Alano, P., Goldring, J.P., Whittle, C., Patterson, S., Chakrabarti, D., and Doerig, C. (2005). PfPK7, an atypical MEK-related protein kinase, reflects the absence of classical three-component MAPK pathways in the human malaria parasite Plasmodium falciparum. Mol. Microbiol. *55*, 184–186.

Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., Moch, J.K., Muster, N., Sacci, J.B., Tabb, D.L., et al. (2002). A proteomic view of the Plasmodium falciparum life cycle. Nature *419*, 520–526.

Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., Sinden, R.E., Waters, A.P., and Janse,

C.J. (2004). A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol. Biochem. Parasitol. *137*, 23–33.

Hall, N., Karras, M., Raine, J.D., Carlton, J.M., Kooij, T.W., Berriman, M., Florens, L., Janssen, C.S., Pain, A., Christophides, G.K., et al. (2005). A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science *307*, 82–86.

Hayward, R.E., DeRisi, J.L., Alfadhli, S., Kaslow, D.C., Brown, P.O., and Rathod, P.K. (2000). Shotgun DNA microarrays and stage-specific gene expression in Plasmodium falciparum malaria. Mol. Microbiol. *35*, 6–14.

Hopkins, J., Fowler, R., Krishna, S., Wilson, I., Mitchell, G., and Bannister, L. (1999). The plastid in Plasmodium falciparum asexual blood stages: a three-dimensional ultrastructural analysis. Protist *150*, 283–295.

Horrocks, P., Dechering, K., and Lanzer, M. (1998). Control of gene expression in Plasmodium falciparum. Mol. Biochem. Parasitol. *95*, 171–181.

Inaba, K. (2003). Molecular architecture of the sperm flagella: molecules for motility and signaling. Zoolog. Sci. *20*, 1043–1056.

Janse, C.J., and van Vianen, P.H. (1994). Flow cytometry in malaria detection. Methods Cell Biol. *42*, 295–318.

Janse, C.J., and Waters, A.P. (1995). Plasmodium berghei: The application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitol. Today *11*, 138–143.

Janse, C.J., and Waters, A.P. (2004). Sexual development of malaria parasites. In Malaria Parasites: Genomes and Molecular Biology, A.P. Waters and C.J. Janse, eds. (Wymondham, United Kingdom: Caister Academic Press), pp. 445–474.

Janse, C.J., Mons, B., Rouwenhorst, R.J., van der Klooster, P.F., Overdulve, J.P., and van der Kaay, H.J. (1985). In vitro formation of ookinetes and functional maturity of Plasmodium berghei gametocytes. Parasitology *91*, 19–29.

Janse, C.J., van der Klooster, P.F., van der Kaay, H.J., van der Ploeg, M., and Overdulve, J.P. (1986a). DNA synthesis in Plasmodium berghei during asexual and sexual development. Mol. Biochem. Parasitol. *20*, 173–182.

Janse, C.J., van der Klooster, P.F., van der Kaay, H.J., van der Ploeg, M., and Overdulve, J.P. (1986b). Rapid repeated DNA replication during microgametogenesis and DNA synthesis in young zygotes of Plasmodium berghei. Trans. R. Soc. Trop. Med. Hyg. *80*, 154–157.

Janse, C.J., van Vianen, P.H., Tanke, H.J., Mons, B., Ponnudurai, T., and Overdulve, J.P. (1987). Plasmodium species: flow cytometry and microfluorometry assessments of DNA content and synthesis. Exp. Parasitol. *64*, 88–94.

Janse, C.J., Boorsma, E.G., Ramesar, J., van Vianen, P., van der Meer, R., Zenobi, P., Casaglia, O., Mons, B., and van der Berg, F.M. (1989). Plasmodium berghei: gametocyte production, DNA content, and chromosome-size polymorphisms during asexual multiplication in vivo. Exp. Parasitol. *68*, 274–282.

Kaslow, D.C. (1997). Transmission-blocking vaccines: uses and current status of development. Int. J. Parasitol. *27*, 183–189.

Lasonder, E., Ishihama, Y., Andersen, J.S., Vermunt, A.M., Pain, A., Sauerwein, R.W., Eling, W.M., Hall, N., Waters, A.P., Stunnenberg, H.G., and Mann, M. (2002). Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. Nature *419*, 537–542.

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.

Lewis, T.S., Shapiro, P.S., and Ahn, N.G. (1998). Signal transduction through MAP kinase cascades. Adv. Cancer Res. *74*, 49–139.

Lu, K.P., and Hunter, T. (1995). The NIMA kinase: a mitotic regulator in Aspergillus nidulans and vertebrate cells. Prog. Cell Cycle Res. *1*, 187–205.

Mallik, R., and Gross, S.P. (2004). Molecular motors: strategies to get along. Curr. Biol. *14*, R971–R982.

O'Connell, M.J., Krien, M.J., and Hunter, T. (2003). Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol. *13*, 221–228.

Ostrowski, L.E., Blackburn, K., Radde, K.M., Moyer, M.B., Schlatzer, D.M., Moseley, A., and Boucher, R.C. (2002). A proteomic analysis of human cilia: identification of novel components. Mol. Cell. Proteomics *1*, 451–465.

Rawlings, D.J., Fujioka, H., Fried, M., Keister, D.B., Aikawa, M., and Kaslow, D.C. (1992). Alpha-tubulin II is a male-specific protein in Plasmodium falciparum. Mol. Biochem. Parasitol. *56*, 239–250.

Sinden, R.E. (1991). Mitosis and meiosis in malarial parasites. Acta Leiden. *60*, 19–27.

Sinden, R.E., Alavi, Y., Butcher, G.A., Dessens, J.T., Raine, J.D., and Trueman, H.E. (2004). Ookinete cell biology. In Malaria Parasites: Genomes and Molecular Biology, A.P. Waters and C.J. Janse, eds. (Wymondham, United Kingdom: Caister Academic Press), pp. 475–500.

Vaidya, A.B., Morrisey, J., Plowe, C.V., Kaslow, D.C., and Wellems, T.E. (1993). Unidirectional dominance of cytoplasmic inheritance in two genetic crosses of Plasmodium falciparum. Mol. Cell. Biol. *13*, 7349–7357.

van Dijk, M.R., Janse, C.J., Thompson, J., Waters, A.P., Braks, J.A., Dodemont, H.J., Stunnenberg, H.G., van Gemert, G.J., Sauerwein, R.W., and Eling, W. (2001). A central role for P48/45 in malaria parasite male gamete fertility. Cell *104*, 153–164.

van Spaendonk, R.M., Ramesar, J., van Wigcheren, A., Eling, W., Beetsma, A.L., van Gemert, G.J., Hooghof, J., Janse, C.J., and Waters, A.P. (2001). Functional equivalence of structurally distinct ribosomes in the malaria parasite, Plasmodium berghei. J. Biol. Chem. *276*, 22638–22647.

Ward, P., Equinet, L., Packer, J., and Doerig, C. (2004). Protein kinases of the human malaria parasite Plasmodium falciparum: the kinome of a divergent eukaryote. BMC Genomics *5*, 79.

Williamson, K.C., Keister, D.B., Muratova, O., and Kaslow, D.C. (1995). Recombinant Pfs230, a Plasmodium falciparum gametocyte protein, induces antisera that reduce the infectivity of Plasmodium falciparum to mosquitoes. Mol. Biochem. Parasitol. *75*, 33–42.