**Plasmodium falciparum** Gametocyte Development 1 (*Pfgdv1*) and Gametocytogenesis Early Gene Identification and Commitment to Sexual Development

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**Abstract**

Malaria transmission requires the production of male and female gametocytes in the human host followed by fertilization and sporadic development in the mosquito midgut. Although essential for the spread of malaria through the population, little is known about the initiation of gametocytogenesis in *vivo* or in *vitro*. Using a gametocyte-defective parasite line and genetic complementation, we show that *Plasmodium falciparum* gametocyte development 1 gene (*Pfgdv1*), encoding a peri-nuclear protein, is critical for early sexual differentiation. Transcriptional analysis of *Pfgdv1* negative and positive parasite lines identified a set of gametocytogenesis early genes (*Pfgc*) that were significantly down-regulated (>10 fold) in the absence of *Pfgdv1* and expression was restored after *Pfgdv1* complementation. Progressive accumulation of *Pfgc* transcripts during successive rounds of asexual replication in synchronized cultures suggests that gametocytes are induced continuously during asexual growth. Comparison of *Pfgc* gene transcriptional profiles in patient samples divided the genes into two groups differing in their expression in mature circulating gametocytes and providing candidates to evaluate gametocyte induction and maturation separately *in vivo*. The expression profile of one of the early gametocyte specific genes, *Pfgc1*, correlated significantly with *Pfgdv1* expression, which is consistent with the ongoing induction of gametocytogenesis in asexual growth and reinforces the need for sustained transmission-blocking strategies to eliminate malaria.

**Citation:** Eksi S, Morahan BJ, Haile Y, Furuya T, Jiang H, et al. (2012) *Plasmodium falciparum* Gametocyte Development 1 (*Pfgdv1*) and Gametocytogenesis Early Gene Identification and Commitment to Sexual Development. PLoS Pathog 8(10): e1002964. doi:10.1371/journal.ppat.1002964

**Editor:** Thomas J. Templeton, Weill Cornell Medical College, United States of America

**Received** May 2, 2012; **Accepted** August 27, 2012; **Published** October 18, 2012

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**Funding:** This investigation received financial support from Public Health Service grants AI40592 and AI48826 from the National Institute of Allergy and Infectious Diseases (NIAID) and the Division of Intramural Research, NIAID, National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

The spread of malaria, a disease that continues to be responsible for 200–300 million clinical cases and the deaths of ~0.7 million people each year, requires the production of gametocytes for transmission from person to person via a mosquito [1,2]. Importantly, it has been shown that gametocytes are not effectively cleared by common antimalarial drugs and therefore allow transmission to mosquitoes despite clearance of asexual parasites [3]. Thus, as efforts toward malaria elimination and eradication progress, the need to understand and monitor the dynamics of the infectious reservoir intensifies.

Within the human host, intraerythrocytic parasites can either replicate asexually or differentiate into a single male or female gametocyte. After release from an infected liver cell, merozoites invade red blood cells (RBC) and the resulting ring stage parasites develop into schizonts containing 16–32 merozoites that are released into the circulation and can each invade a new RBC to begin the cycle again. For transmission to mosquitoes, a subpopulation of schizonts produce merozoites that invade RBC and each differentiate over the course of 10–12 days into a single male or female gametocyte [4–8]. After being taken up in a blood meal by a mosquito, gametocytes are stimulated to produce gametes, mate and develop into sporozoites that are infectious to humans during subsequent blood-feeding. Despite the importance of gametocytogenesis in the propagation of infection, the nature and timing of its induction is unknown [9–11]. Specifically, the lack of markers for early sexual stages, gametocyte-committed schizonts and the ring-stage equivalent of sexually committed parasites has made it difficult to determine whether gametocyte production is a one-time stimulus-induced event or a continuous process with a subpopulation of parasites converting to sexual development during each cycle [10,12,13]. This has important ramifications for control strategies as continuous gametocyte...
Author Summary

As malaria control efforts move toward eradication it becomes increasingly important to develop interventions that block transmission. Consequently, advances are needed in our understanding of the production of gametocytes, which are required to transmit the disease. This report provides a first view of the initial stages of gametocytogenesis in vivo and in vitro and demonstrates that during each asexual replication cycle a subpopulation of parasites convert to gametocyte development providing a long transmission window. We also identify a gene that is critical for gametocyte production, *P. falciparum* gametocyte development 1 (Pfgdv1) and a set of genes specifically expressed during early gametocytogenesis in *P. falciparum* (Pfge genes). The expression profile and perinuclear location of Pfgdv1 in a subpopulation of schizonts is consistent with a role in an early step in gametocytogenesis. The RNA levels of Pfgdv1 and the Pfge genes accumulated gradually over several asexual cycles in vitro suggesting ongoing gametocyte formation during asexual growth. The further evaluation of these genes in a cohort of malaria infected patients indicated they are good candidates for markers to distinguish ring stage parasites committed to gametocyte production from circulating mature gametocytes, allowing direct analysis of the initiation of sexual differentiation in vivo.

production would significantly prolong the transmission window. In addition to the lack of early gametocytogenesis markers, the analysis of *P. falciparum* gametocyte development is complicated by sequestration of both mature asexual parasites (trophozoites and schizonts) and immature gametocytes (stages II to IV) in vasculature during human infection, which means that only rings, including those committed to sexual development, and mature gametocytes are detected in peripheral blood [13].

To better define gametocyte commitment and evaluate the timing of induction, we compared clonal parasite lines that differed in their ability to produce gametocytes to identify the genes that contribute to gametocytogenesis. Genetic complementation confirmed the important role of one of the identified genes, *P. falciparum* gametocyte development 1 gene (Pfgdv1) in gametocyte production and the expression of a set of *P. falciparum* gametocytogenesis early genes (Pfge). The transcription profiles of these genes were then used to investigate gametocyte induction through sequential asexual cycles in vitro demonstrating the continuous production of gametocytes. The analysis was also extended to clinical samples and suggests that there are distinct molecular signatures for mature gametocytes and early committed gametocytes in patient blood samples that could be used in future studies to differentiate gametocyte induction from maturation. This work describes the first direct analysis of this critical early step toward malaria transmission and identifies markers to continue to probe sexual differentiation in vivo and in vitro.

Results

Identification of an early *P. falciparum* gametocyte-specific transcriptome

To identify genes that play an important role in the initial induction of gametocytogenesis, we compared the transcriptional profile of a set of clonal gametocyte-over-producing (3D7.G+, average peak gametocytemia ± SEM, 6.97±2.72%) and gametocyte-deficient (3D7.Gdef, average peak gametocytemia ± SEM, 0.009±0.009%) parasite lines. Both lines, 3D7.G+ and 3D7.Gdef, were cloned from *P. falciparum* strain 3D7 following targeted disruption of Pf230 (PFB0405w) [14,15]. RNA was isolated from tightly synchronized cultures on day 4 of gametocyte induction (at parasitemias of 1.4% for 3D7.G+ and 0.9% for 3D7.Gdef) and then again 2 days later at parasitemias of 3.2% and 3.5%, respectively, and was hybridized to a 70-mer oligonucleotide array representing 5,692 *P. falciparum* genes [16]. Both time points were early in gametocytogenesis, prior to detection of stage II gametocytes in Giemsa-stained culture smears, which in previous studies was the first gametocyte-specific time point for the identification of gametocyte-specific transcripts [17]. Using a 10 fold signal difference as a threshold, 11 genes were differentially regulated at both time points (Fig. 1) and an additional 9 genes had a≥5 fold signal difference (p<0.05) (Table S1). They include seven known gametocyte specific genes Pf27, Pf4, Pf14.744, Pf14.748, Pf47, Pfmdv1, and Pfge12 [18–23]. These seven genes, as well as PF11_0038, PF14_0290, PF14_0588, PF14_0708 and PF14_0735 have also been identified by proteomic analysis of stage I/II gametocytes; while the remaining genes have not been associated with gametocytogenesis [24]. The differential expression patterns of the 11 genes were confirmed using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) or northern blots and were designated as *P. falciparum* gametocytogenesis early (Pfge) 1 to 11 according to their 3D7.G+/3D7.Gdef signal ratio at the first time point (Fig. 1 and Table S1). This name will be used for those genes without a common name; for example, PF14_0744 had the highest signal ratio and was named Pfge1. Pfge genes 1–8 are located in subtelomeric regions (<150 kb from the telomere) and, except for Pfge9, only have close orthologs (Blast E score≤10−35) in *P. reichenowi*. None of the Pfge genes were predicted to have signals for secretion and/or export to the erythrocyte, which was consistent with gametocyte-specific modifications of the external environment being an early step in sexual differentiation.

Defective gametocytogenesis is linked to deletion of a locus encoding Pfgdv1

To study the genetic basis for the inability of the Gdef line to make gametocytes, we used two *P. falciparum* microarrays, one with ~7000 70-mer oligonucleotides and the other with 2.5 million 25-mer tiling oligonucleotides, to compare genomic DNA (gDNA) from the 3D7.G+ and 3D7.Gdef lines [16,25,26]. A single oligonucleotide, i13417_1 (MAL9: 1,379,276–1,379,345) designated with an asterisk in Fig. 2A, had a significantly lower signal when the 70-mer oligo array was hybridized with gDNA from the 3D7.Gdef clone than with that from the 3D7.G+ clone, suggesting substitution(s) or a deletion in the 3D7.Gdef genome sequence. Two oligonucleotides (i14759_1 and i12812_3) flanking the i13417_1 probe hybridized to gDNA from both parasite clones with similar intensities. This hybridization data defined a DNA segment of ~30 kb on chromosome 9 that was likely deleted in the 3D7.Gdef clone (Fig. 2A). To confirm this observation, we designed eight PCR primer pairs to amplify the region between the two positive oligonucleotides (Fig. 2A). PCR products were obtained for all eight primer-pairs using gDNA from 3D7.G+ and wild type (WT) 3D7 lines, but only primer pairs 1, 7, and 8 amplified products from 3D7.Gdef gDNA (Fig. 2B). DNA sequencing of a PCR product spanning the 3D7.Gdef deletion defined the breakpoints to an 18,920 bp region (1,374,382 and 1,393,302 bp) on chromosome 9. The deletion is flanked by polyA tracts of >18 bp that have been reported to be sites of frequent recombination and deletion [27].

This region of chromosome 9 was intact in a second gametocyte-producing strain (Indochina), but was absent in two other gametocyte-deficient laboratory lines derived from FCR3
Figure 1. Expression analysis of the 3D7.G+ and 3D7.G-def clones identifies *P. falciparum* gametocytogenesis early genes (*Pfge*). Mean ratios of microarray signals (mean±SEM) obtained from the 3D7.G+ and 3D7.G-def clones at 1.4/0.9% parasitemia (dark gray bar) and at 5.2/5.5% parasitemia (light gray bar), respectively, for the 11 *Pfge* genes with a ratio of 3D7.G+/3D7.G-def>10 are plotted in descending order. *Pfge1* (PFI1710w) had the fourth highest ratio and is indicated by an asterisk (*). Previously described *Pfge* genes [17,20–22,82], are denoted with an asterisk (*) and a vertical line indicates a gene represented by two oligonucleotides. *Pfge* number, PlasmoDB ID, and common name are listed on the right. The central panel is a summary of the characteristics of the *Pfge*-dependent genes. The first row (L) indicates whether the gene is subtelomeric (<150 kb from the telomere) (gray square) or within a region of the chromosome that has synteny with other species (black square) [83]. The panel on the left indicates whether the gene encodes a secretory signal sequence (S, black square), PEXEL/HLS export domain (E, black square) or transmembrane domain (T, black square).

doi:10.1371/journal.ppat.1002964.g001

and HB3 (designated FCR3.G_def and HB3.G_def) [Fig. 2B]. All the parasite lines retained region 1, but regions 2 to 7 were absent in both FCR3.G_def and HB3.G_def [Fig. 2B]. These results were consistent with a previous observation of a loss of both gametocyte production and cytoadherence to cells expressing CD36 after a frequent loss of this region in culture, it appears to be retained in the deletion varies between strains [28–31]. Although there is a deletion at one end of chromosome 9 and showed that the size of the deletion varies between strains [28–31]. Although there is a break in synteny with the rodent genome sequences in field isolates consistent with a role in gametocyte production which is required for transmission in the field, but not for asexual growth in culture [30,32].

To further investigate whether there were changes in the genome other than the chromosome 9 deletion in the 3D7 derived clones, we used a high-density tiling array (PFSANGER GeneChip) [26] to scan the parasite genome. Comparison of gDNA isolated from WT 3D7, 3D7.G+, 3D7.G_def and FCR3.G_def confirmed the deletion on chromosome 9 in the gametocyte-deficient parasites (log ratio of 3D7.G_def and FCR3.G_def over WT 3D7 signals were −5.31, p<0.01). The only other difference detected by the tiling array between both the G_def lines and the G+ lines was a 342 bp region in the C-terminus of a 1,092 bp subtelomeric variable open reading frame (*stevor* (MAL13P1.7)) located in the subtelomeric region of chromosome 13. *Stevo*r are highly polymorphic and variations in these loci are common. The original 70-mer microarray did not detect the difference in the *stevor* gene because of the lack of probes in this region.

The 18,920 bp region deleted in 3D7.G_def and the ~26,000 bp region absent in FCR3.G_def contained a single annotated gene (PFI1710w or *Pfge*4 on the original G+/G_def microarray) that was re-named *Pfge4* because of its putative role in *Plasmodium falciparum* gametocyte development. This gene had previously been called cytoadherence-linked protein due to its association with a loss of cytoadherence following a 0.3 Mb deletion of the entire subtelomeric region of chromosome 9 [33], but later analysis of additional lab isolates demonstrated that it was upstream from the cytoadhesion locus [34]. The C-terminal half of the predicted 72-kDa protein encoded by *Pfge4* contains two helix-rich regions (aa 231–312 and 508–577) (Fig. S1). Homologues in *Plasmodium reichenowi*, *Plasmodium vivax*, *Plasmodium knowlesi*, and *Plasmodium gallinaceum* have similar helix-rich domains, and all are predicted to localize to the nucleus by both PSORT and PSORTII algorithms [35–37], although no nucleotide binding domains were detected. There is a break in synteny with the rodent genome sequences in this region and no obvious *Pfge4* homologues are evident.

**Pfge1** complementation restores gametocyte production in a gametocyte-deficient line

To directly test whether *Pfge1* has a role in gametocytogenesis, plasmids containing a blastidicin (BSD) resistance gene, the complete *Pfge1* predicted open reading frame (bp 1-1800) and a segment of the 5′ flanking region (~1236 to −1 bp from the ATG) with or without a 5′ hemagglutinin (HA) epitope tag (pCBM.BSD. *Pfge1*.5′.ORF and pCBM.BSD. *Pfge1*.5′.HA.ORF, respectively) were used to transform the 3D7.G_def clone. Blastidicin-resistant parasites obtained from two independent transformations produced gametocytes in numbers comparable to the WT 3D7 strain and significantly more gametocytes than the uncomplemented G_def line (peak gametocytogenesis 1.6–3.1% for 3D7.G_def, 0.75–3.9% for 3D7.G_def+ *Pfge1*; 1.6–4.3% for WT 3D7, and 0–0.2% for 3D7.G_def+ *Pfge1*; p<0.05, ANOVA followed by Tukey’s multiple comparison) (Fig. 2D–F). In contrast, gametocytogenesis was not restored by transformation of 3D7.G_def with a pCBM.BSD.reporter construct alone (data not shown). Southern blot analysis confirmed that the genomic deletion was still present in the complemented lines, ruling out the possibility of WT parasite contamination (Fig. 2G). DNA fragments from restriction digestion (BsaI) confirmed the presence of the complementation plasmids in the 3D7.G_def+ *Pfge1* and 3D7.G_def+HA. *Pfge1* parasites (1.1 kb and 1 kb, respectively), whereas a 4.4 kb fragment from WT 3D7 and 3D7.G+ parasites was consistent with the presence of a genomic copy of *Pfge1*. The complemented parasites progressed through all five morphological stages of gametocytogenesis, and gametogenesis could be induced by conditions simulating those of the mosquito midgut, 25°C, pH 8.0 and 100 μM xanthine/xanthine oxidase [13]. Further, northern analysis of RNA harvested from WT, G_def and the complemented lines, 3D7.G_def+ *Pfge1* and 3D7.G_def+HA. *Pfge1*, demonstrated that expression of the *Pfge* genes 1–11 as well as Pfs48/49 were restored (Fig. 2G and H).

**Pfge1** overexpression enhances gametocyte production in wild type 3D7 parasites

To further confirm a role for *Pfge1* in the modulation of gametocytogenesis, an additional copy of *Pfge1* was expressed in WT 3D7 parasites using the pCBM.BSD. *Pfge1*.5′.HA.ORF episomal expression construct. As shown in Fig. 2I, transformed lines expressing a second copy of *Pfge1* attained a higher mean
A. Chr9
1,374,582 1,393,502
1368k

B. PCR 1 2 3 4 5 6 7 8 G’cytemia
Indochina 0.9 / 1.1 %
3D7.G_{def} 0 / 0.05 %
FCR3.G_{def} 0 / 0 %
HB3.G_{def} 0 / 0 %

C. + d a b WT
Pfgdv1 -4.4 kb
Pfg27 -1 kb

D. Pfgdv1
Gametocytemia (%) vs. Day

E. HA.Pfgdv1
Gametocytemia (%) vs. Day

F. WT
HA.Pfgdv1
G_{def}

G. w d a d b w d a d b w d a b w d a b w d a b

H. WT a b
αPfs48/45

I. Gametocytemia %

P. falciparum gametocytogenesis
gametocytogenesis than the parental WT 3D7 and the G_strain line (WT, 1.2±0.36; WT+HA_PfPgdv1, 4.65±1.58; G_strain, 0±0; mean ± SEM, p<0.05 ANOVA) followed by Tukey’s multiple comparison test). Together, these results demonstrate that PfPgdv1 plays a key role in P. falciparum gametocytogenesis.

PfPgdv1 localizes to the nuclear periphery

The subcellular location of PfPgdv1 was evaluated by tagging the gene with green fluorescent protein (GFP) or a HA epitope. Consistent with the nuclear localization predicted by PSORT, both tagged proteins had a punctate expression pattern around the nuclear periphery in a subpopulation (~0.2–0.4%) of trophozoites and schizonts (Fig 3A). Parasites expressing GFP-tagged PfPgdv1 also stained with anti-sera against early gametocyte markers PfP16 (PfGE11) and PfP14.748 (PfGE3), suggesting that these parasites were committed to gametocytogenesis (Fig 3B) [19,38]. The major difference between NAG-treated and untreated cultures was the third schizont peak on day 7. In both cases the asexual parasitemia declined by day 8 and a similar number of ring stage parasites and gametocytes were produced in both conditions. These findings suggest that gametocytogenesis was induced prior to the death of the schizonts on day 7 and was not triggered directly by the conditions that cause the natural decline in asexual growth (crash). The gametocyte conversion rate was estimated to be 13.8% and 13% in NAG treated and untreated cultures, respectively, by using the daily RBC counts and Giemsa-stained culture smears to calculate the total number of gametocytes and ring stage parasites produced over the first 10 days of culture.

Expression profiles of PfPgdv1 and the Pfge genes identify an early gametocyte committed form (G_cyc) during the transition from asexual to sexual differentiation

Development of sensitive molecular assays that distinguish committed gametocytes in the presence of asexual parasites should facilitate monitoring the transition from asexual to sexual differentiation. The identification of PfPgdv1 and the Pfge genes that are selectively expressed early in gametocyte-committed (G_cyc) parasites may allow the identification of a sub-population of committed cells that have not yet acquired the morphological characteristics of Gtv gametocytes. To further evaluate PfPgdv1 and the Pfge expression patterns as markers of gametocyte commitment, we followed emergence of the G_cyc population over multiple asexual cycles in synchronized in vitro cultures using RT-qPCR and compared the expression patterns with constitutively- and asexual-stage specifically expressed genes.

Strain 3D7 P. falciparum gametocyte cultures were initiated with 0.1% synchronized ring stage parasites and followed daily for 2 weeks, through 3 asexual cycles (1, 2, 3, Fig 4A and B). Asexual parasitemia peaked in 6–7 days with a subsequent decline after day 7 and a rise in Giemsa-detectable stage II gametocytes that peaked on day 10. In one set of cultures (Fig 4A), N-acetyl-D-glucosamine (NAG) was added on day 6–8 when the ring stage parasitemia reached 4% to prevent schizont maturation and merozoite invasion as previously described [41,42]. In the absence of NAG (Fig 4B), ring forms observed on day 6 developed into schizonts by day 7, but did not form new ring stage parasites, causing the parasitemia to decline rapidly by day 8. Consequently, the major difference between NAG-treated and untreated cultures was the third schizont peak on day 7. In both cases the asexual parasitemia declined by day 8 and a similar number of ring stage parasites and gametocytes were produced in both conditions. These findings suggest that gametocytogenesis was induced prior to the death of the schizonts on day 7 and was not triggered directly by the conditions that cause the natural decline in asexual growth (crash). The gametocyte conversion rate was estimated to be 13.8% and 13% in NAG treated and untreated cultures, respectively, by using the daily RBC counts and Giemsa-stained culture smears to calculate the total number of gametocytes and ring stage parasites produced over the first 10 days of culture.

The expression profiles of Pfge genes, the gene encoding knob associated histidine rich protein (PfKnob, PFB0100c, ring specific), and the gene encoding aldolase (PF14_0425, housekeeping gene) were determined using RT-qPCR in three independent experiments. The data was analyzed using ΔΔ cycle threshold (C_T) relative quantitation [43] and plotted as the log2 of the relative abundance with day 2 as a reference and the housekeeping gene seryl tRNA synthetase (PF07_0073) as the constitutive control (Fig 4C–F) [44]. Peaks in PfPgdv1 transcript levels corresponded with the peaks of schizont stage parasites, but were negatively correlated with ring stage peaks and the transcript levels of PfKnob and PfPge before day 8. Additionally with subsequent asexual cycles PfPgdv1 RNA levels progressively increased and continued to be expressed through gametocyte formation (Fig 4C and D; Tables 1 and 2; Fig S2). The transcript levels of only one other Pfge gene, PfPmhp7-5 also peaks in schizonts (Fig 4C and D),
whereas the rest of the Pfge gene transcripts gradually increased as more parasites switched to gametocytes during each asexual cycle. If we consider the expression of Pfgdv1 as occurring very early in sexual development, then the results suggest that a subset of schizonts are already committed on day 3 prior to a rise in parasitemia. It has previously been reported that some schizonts are committed to gametocyte production [4–8], but no genes have been linked to this to date and the timing has not been evaluated.

Transcript levels peaked on day 8 for all the Pfge genes in Fig. 4E and F, except Pf47 which continued to rise through day 12.

**Pfgdv1 and Pfge gene expression profiles in blood samples from malaria patients**

The identification of genes that are specific for gametocyte-committed ring stage parasites provides an opportunity to evaluate whether these stages can be detected circulating in malaria patients and therefore be developed as biomarkers for gametocyte induction in vivo. Markers specific for early gametocytes could then be paired with mature stage V gametocytes markers, such as Pf25 [45], for use in future clinical studies to evaluate the parameters that affect gametocyte initiation and maturation. Since patient blood samples can contain both ring stage parasites and mature gametocytes it is important to identify genes that are expressed only in committed ring stage parasites, not mature gametocytes. *In vitro* culture is not a good model for this because it is difficult to separate immature from mature gametocytes, therefore we directly evaluated the expression profile of Pfgdv1 and the Pfge genes in a microarray analysis of samples obtained from 20 patients on the Thailand Myanmar border with a range of different gametocytemias and asexual parasitemias (Fig. 5 and Table S2).

Cluster analysis of the expression profiles separated the genes into 2 major groups. One group included Pf47, Pf48, Pf43 and Pfgdv1, as well as late gametocyte markers Pf25 and Pf28 and had higher expression levels in patient samples with higher gametocytemias (p = 0.0185 Mann Whitney) limiting their usefulness as biomarkers for gametocyte committed rings in field samples. The other group could be subdivided into 2 clusters, one containing Pfgeo, Pfmsp7-5 and Pfge7 and another containing Pfgdv1, Pfge2 and Pfge7. Significantly, the expression profile of Pfgdv1 correlated with asexual parasitemia even when corrected for false discovery (FDR) and controlled for gametocytemia (ANCOVA analysis, correlation = 0.667, p = 0.002, FDR q-value = 0.010) (Fig. 5). Given the previous data establishing the specificity of these genes for early gametocytes [19] and their *in vitro* expression pattern (Fig. 4E and F), these results suggest that they are good candidates for further evaluation as markers for different stages of gametocyte commitment in the field. The close correlation
between the expression of Pfge1 and asexual parasitemia is also consistent with the ongoing induction of gametocytes during asexual growth that was observed in vitro and should be analyzed in future field studies.

**Discussion**

The present study identified a *P. falciparum* gene, Pfgdv1, that plays a role in early gametocytogenesis. A set of downstream genes involved in early events in sexual development were also identified, and together these permitted analysis of gametocyte induction in synchronized cultures and patient samples. The role of Pfgdv1 in gametocytogenesis was demonstrated through four lines of evidence, 1) the association of a genomic deletion containing Pfgdv1 in 3 spontaneously occurring gametocyte-deficient lines; 2) restoration of gametocyte production following complementation of a 3D7 gametocyte-deficient line with WT Pfgdv1; 3) enhancement of gametocyte production in WT parasites following overexpression of Pfgdv1 using an episomal copy of the gene and 4) a peri-nuclear location of epitope tagged-PfGDV1 in a subpopulation of schizonts, as well as early pre stage I gametocytes co-expressing Pf16 and Pfge3. Molecular complementation is an important control for inadvertent mutations, and this is the first report of restoration of gametocyte production by complementation of *P. falciparum*. Overexpression studies not only provide evidence that increases in Pfgdv1 activity modulate gametocyte production in a dynamic fashion, but may also provide a molecular tool to augment the production of gametocytes to facilitate the development of transmission-blocking reagents [46]. Previous work demonstrated that episomal expression of a neighboring gene Pfigg (PF11720w) increased Pf16 expression in a non-gametocyte producing line, but did not restore gametocyte production [29]. Pfigg was intact in the *G_ig* line used in this study and expression was not significantly affected by the loss of Pfgdv1; however, it is possible that it could act in conjunction with Pfgdv1 to stimulate gametocytogenesis.
The presence of a Pfgdv1 homologue in the primate malarias P. vivax and P. knowlesi, as well as the avian malaria P. gallinaceum, suggests a shared role in early gametocyte formation since later steps in gametocyte development, such as changes in morphology and the time course of maturation, are quite different between these species [47–49]. The apparent absence of a Pfgdv1 homologue in the rodent malarias is intriguing, given the currently accepted phylogeny that separates avian and mammalian Plasmodium [47–49]. If the gene is not found in the rodent Plasmodium genomes, it is possible that gametocyte induction is distinct in the rodent Plasmodium or that another gene with a similar structure, but a unique amino acid sequence is involved. In contrast to Pfgdv1, eight of the Pfge genes are unique to the Laverania subspecies of P. falciparum, which includes P. falciparum and P. reichenowi and is characterized by morphologically distinct gamocytes that sequester during a prolonged 10–12 day gametocyte maturation period [47]. Seven of these Laverania-specific Pfge genes are predicted to be secreted and exported proteins and could be involved in adaptations to the RBC environment that are required for sequestration and survival over 10–15 days in an immune competent host [19,23,24,50–54].

The lack of expression of the Pfge genes in parasites with defective Pfgdv1 is consistent with Pfgdv1 playing a key upstream role in early gametocytogenesis or commitment to sexual development. The transcription profile of Pfgdv1 is consistent with the observed pattern of PfGDV1 protein expression in a subpopulation of schizonts and continued expression in early Gc parasites. It is tempting to speculate that the peri-nuclear location of PfGDV1 in gametocyte-committed schizonts could indicate a role in the regulation of gametocytogenesis [55,56].

Table 1. Pearson correlation analysis of gene expression profiles in in vitro, NAG-treated cultures.

| NAG    | kahrp | aldo | Pfgdv1 | msp7-5 | Pfge1 | Pfge2 | Pfge7 | Pfge27 | Pfge3 | Pfge8 | Pf16 | Pf47 | geco |
|--------|-------|------|--------|--------|-------|-------|-------|--------|-------|-------|------|------|------|
| kahrp  | 1     |      |        |        |       |       |       |        |       |       |      |      |      |
| aldo   | −0.296| 1    |        |        |       |       |       |        |       |       |      |      |      |
| Pfgdv1 | −0.788| 0.598| 1      |        |       |       |       |        |       |       |      |      |      |
| msp7-5 | −0.326| 0.456| 0.608  | 1      |       |       |       |        |       |       |      |      |      |
| Pfge1  | 0.090 | 0.068| 0.213  | 0.394  | 1     |       |       |        |       |       |      |      |      |
| Pfge2  | −0.277| 0.298| 0.525  | 0.540  | 0.863 | 1    |       |        |       |       |      |      |      |
| Pfge7  | −0.040| 0.005| 0.173  | 0.313  | 0.851 | 0.878| 1     |        |       |       |      |      |      |
| Pfge27 | −0.345| 0.190| 0.479  | 0.481  | 0.838 | 0.959| 0.912 | 1      |       |       |      |      |      |
| Pfge3  | −0.195| 0.344| 0.385  | 0.637  | 0.681 | 0.860| 0.743 | 0.829  | 1    |       |      |      |      |
| Pfge8  | −0.426| 0.170| 0.437  | 0.479  | 0.748 | 0.888| 0.874 | 0.960  | 0.828 | 1    |      |      |      |
| Pf16   | −0.507| 0.039| 0.497  | 0.403  | 0.605 | 0.816| 0.846 | 0.881  | 0.694 | 0.910| 1    |      |      |
| Pf47   | −0.616| 0.256| 0.479  | 0.543  | 0.327 | 0.667| 0.609 | 0.752  | 0.760 | 0.832| 0.800| 1    |      |
| geco   | 0.754 | −0.486| −0.689| −0.060| 0.443 | 0.143| 0.365 | 0.129  | 0.242 | 0.087| −0.055| −0.108| 1    |

The expression profiles of the relative abundance of indicated genes in relation to seryl tRNA synthetase at day 2 were compared to each other in the NAG treated (NAG) the Pearson correlation coefficient is indicated. Representative data shown is from one of 3 independent experiments.

doi:10.1371/journal.ppat.1002964.t001

Table 2. Pearson correlation analysis of gene expression profiles in in vitro untreated cultures.

| No NAG | kahrp | aldo | Pfgdv1 | msp7-5 | Pfge1 | Pfge2 | Pfge7 | Pfge27 | Pfge3 | Pfge8 | Pf16 | Pf47 | geco |
|--------|-------|------|--------|--------|-------|-------|-------|--------|-------|-------|------|------|------|
| kahrp  | 1     |      |        |        |       |       |       |        |       |       |      |      |      |
| aldo   | −0.273| 1    |        |        |       |       |       |        |       |       |      |      |      |
| Pfgdv1 | −0.863| 0.476| 1      |        |       |       |       |        |       |       |      |      |      |
| msp7-5 | −0.423| 0.361| 0.581  | 1      |       |       |       |        |       |       |      |      |      |
| Pfge1  | −0.244| 0.199| 0.371  | 0.626  | 1     |       |       |        |       |       |      |      |      |
| Pfge2  | −0.445| 0.253| 0.622  | 0.567  | 0.874 | 1    |       |        |       |       |      |      |      |
| Pfge7  | 0.011 | −0.035| 0.232  | 0.321  | 0.782 | 0.853| 1     |        |       |       |      |      |      |
| Pfge27 | −0.379| 0.237| 0.556  | 0.569  | 0.870 | 0.975| 0.862 | 1      |       |       |      |      |      |
| Pfge3  | −0.315| 0.204| 0.435  | 0.527  | 0.829 | 0.891| 0.820 | 0.940  | 1    |       |      |      |      |
| Pfge8  | −0.182| 0.053| 0.359  | 0.289  | 0.639 | 0.800| 0.825 | 0.781  | 0.855 | 1    |      |      |      |
| Pf16   | −0.339| −0.063| 0.513  | 0.347  | 0.658 | 0.871| 0.890 | 0.846  | 0.811 | 0.878 | 1    |      |      |
| Pf47   | −0.344| 0.087| 0.449  | 0.394  | 0.462 | 0.755| 0.686 | 0.751  | 0.770 | 0.818 | 0.826| 1    |      |
| geco   | 0.583 | −0.546| −0.632 | −0.025| 0.317 | 0.076| 0.416 | 0.193  | 0.273 | 0.171 | 0.119| 0.060| 1    |

The expression profiles of the relative abundance of indicated genes in relation to seryl tRNA synthetase at day 2 were compared to each other in the untreated cultures (No NAG) and the Pearson correlation coefficient is indicated. Representative data shown is from one of 3 independent experiments.

doi:10.1371/journal.ppat.1002964.t002
homology with known transcription factors or nucleotide-binding proteins suggests that instead of a direct role in gene expression, PfGDV1 could be part of a regulatory complex. Developmental switches often include a number of scaffold and regulatory proteins, which could serve as a model for PfGDV1. Examples in yeast include Far1 that binds Cdc28-Cln2 kinase causing cell cycle arrest [57] and Rmf1 that tethers histone deacetylase Hst1 and the DNA-binding protein Sum1 [58]. As the first protein found to localize to the nucleus during sexual differentiation, PfGDV1 provides a starting point for identifying the additional functional components of the regulatory cascade in the sexual development of P. falciparum.

The expression of Pfgdv1 and the Pfgene genes through multiple rounds of asexual growth of strain 3D7 parasites and continued expression for 24 hours after the clearance of asexual parasites is in marked contradiction to the hypothesis that gametocyte differentiation is induced by the same stress imposed by high density culture that inhibits further asexual growth. Additionally, blocking the development of high parasitemia on day 7 using NAG did not affect gametocyte production. However, other parasite strains will have to be tested to determine if this is a general characteristic. These data suggest that in strain 3D7 parasites, a subpopulation of parasites convert to gametocytogenesis during each asexual cycle, instead of a model where the majority of parasites differentiate in response to a one-time stimulation event as seen in yeast after exposure to stress-induced mating pheromone, a quorum response in bacteria to high cell levels, or induction factor stimulation of trypanosomes [13,59–62]. The ongoing conversion of a subpopulation of intraerythrocytic parasites to sexual differentiation during each asexual cycle fits well with the pattern of gametocyte production observed in neurosyphilis patients receiving malaria therapy [63,64]. During the long course of malaria therapy, the rise of circulating stage V gametocytes follows the same pattern as the increase in asexual parasitemia, but is delayed by the 10 days required for gametocyte maturation and plateaus at ~10% of the maximal asexual parasitemia. These findings suggest that during each asexual cycle ~10% of the parasites are committed to gametocytogenesis (Fig. 6). This ratio is similar to the gametocyte conversion rate reported for P. vivax [65], and the ratio of ring stage parasites to stage II gametocytes observed in our in vitro cultures (13.35% ± 0.175, mean ± SEM), suggesting a consistent sexual induction rate in vitro and in vivo. A model of continuous gametocyte induction during each asexual erythrocytic cycle is also supported by the significant correlation
found between Pfge1 expression levels with asexual parasitemia in patients in the current study and previous reports that gametocytes can be detected in >90% of malaria patients when analyzed by quantitative-nucleic acid sequence-based amplification [63,64]. The continuous differentiation of a subpopulation of progeny from a reservoir of self-renewing stem cells seen in sperm formation or hematopoiesis may be a better model for gametocytogenesis than sexual differentiation in yeast [66,67]. Such a strategy of ongoing gametocyte production could facilitate transmission by providing a continuous source of infectious gametocytes whenever a mosquito takes a blood meal.

Concurrent gametocyte induction and asexual growth does not rule out modulation of gametocyte production by environmental conditions or host/parasite genetics. As demonstrated here, gametocyte production was enhanced by Pfge1 overexpression in WT parasites and other studies show that different parasite lines produce different numbers of gametocytes and gametocyte yields vary in different batches of in vitro culture [61,68,69]. Again, this type of modulation could be similar to hematopoiesis where the cytokine milieu influences the number and type of blood cells produced by altering maturation without stimulating all the stem cells to differentiate, thus maintaining an asexually replicating stem cell stock [67]. In humans, variation in gametocyte levels have been associated with anemia (<30 hematocrit), duration of illness and drug treatment, but whether the changes were due to differences in gametocyte induction or maturation was not evaluated [70–73]. The identification of these Pfge genes provides new tools to evaluate gametocytogenesis directly in humans. If confirmed, the continuous commitment to gametocytogenesis suggests a long transmission window during human infections and reinforces the need to include these essential stages of the parasite life cycle in malaria control efforts. It also suggests the importance of developing methods that provide widespread, long lasting protection, such as vaccines or drug combinations that are effective against all erythrocytic stages.

Materials and Methods

Ethics statement

All research involving human subjects was reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army and have been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was provided by study participants and/or their legal guardians.

P. falciparum parasite strains

P. falciparum strain 3D7 was provided by D. Keister (National Institute of Allergy and Infectious Diseases). P. falciparum parasites FCR3.Gdef, HB3.Gdef and Indochina were provided by M. Klemba (Virginia Institute of Technology). WT 3D7 was transformed with pDT.Tg23.230-D1.356 (pD1.356) to study the role of gamete surface antigen Pf230, and two independently transformed, pyrimethamine-resistant lines were obtained as described [14]. One clonal line produced gametocytes (3D7.G+), whereas the other was gametocyte-deficient (3D7.Gdef), neither expressed full length Pf230. Parasites were synchronized using sorbitol treatment or MACS columns (Miltenyi Biotec, Auburn, CA), and gametocyte cultures were set up at 0.1% ring stage parasitemia [19,74]. Parasite stages were quantitated using Giemsa-stained culture smears to determine parasitemia and total red blood cell counts.

DNA and RNA isolation and analysis

Parasite gDNA was isolated using the Wizard gDNA Purification kit (Promega, Madison, WI), and RNA was isolated from parasites at the indicated developmental stage using TRIzol (Life Technologies, Grand Island, NY) as described [19]. For Southern and northern analyses, gDNA digested with the indicated restriction enzymes (New England Biolabs, Ipswich, MA) or

Figure 6. Model for continuous gametocytogenesis. During each round of the asexual cycle, a proportion of the schizonts (Sc, light blue) produced are committed to producing merozoites (Mc, light blue) that will differentiate into gametocytes (Gc, G I–V) after invading a RBC. The expression profile for Pfge1 is indicated by a line under the corresponding stage.

doi:10.1371/journal.ppat.1002964.g006
RNA samples were size-fractionated and transferred to Nytran membrane (Schleicher and Schuell, Keene, NH), respectively. The membranes were incubated with randomly primed 32P-dCTP-labeled probe in 1x DIG hybridization buffer (Roche, Indianapolis, IN) at 65°C overnight, washed twice in 0.2x SSPE (30 mM NaCl, 2 mM NaN3PO4, 0.2 mM EDTA) and 0.5% Sodium dodecyl sulfate for 30 min at 65°C, and visualized by autoradiography.

Microarray analysis

Parasite gDNA. Two different platforms were used to analyze parasite gDNA: A) the 70-mer oligonucleotide array designed by the DeRisi laboratory (Operon Technologies, Alameda, CA) [16] and B) the PFSANGER GeneChip (Affymetrix, Inc., Santa Clara, CA) designed at the Sanger Center [26]. For the 70-mer oligonucleotide array, fluorescently labeled probes were generated from parasite gDNA by random prime labeling with Cy5- or Cy3-UTP, in a 30 μl reaction containing 30 mM Tris, pH 6.8, 5 mM MgCl2, 100 μM EDTA, 10 mM 2-mercaptoethanol, 300 μg/ml random hexamer, 120 μM each dATP, dGTP, and dCTP, 60 μM dUTP, 60 μM Cy5-dUTP or Cy3-dUTP, and 50 U Klenow fragment. The mixture was incubated at 37°C for 2 h and then stopped by increasing EDTA to 50 mM. Labeled probes were purified by filtering through six successive washes in TE, pH 8.0 (10 K MWCO Vivaspin 500, Vivasience AG, Hanover, Germany) and used in the MAUI hybridization system (BioMicro Systems, Salt Lake City, UT). Array hybridization was performed by the Genomic Technologies Section (National Institutes of Health, Bethesda, MD) with a GenePix 4000B array scanner (Axon Instruments, Union City, CA). The microarray signals from each array spot were normalized so that the median Cy5:Cy3 ratio was set as 1.0. Collected data were analyzed with GenePix Pro 4.0 (Axon Instruments) and web-based microarray tools (mAsk) developed by the Bioinformatics and Molecular Analysis Section, Center for Information Technology, NIH [https://maadb.niaid.nih.gov]. For the PFSANGER GeneChip, parasite gDNA was fluorescently labeled as described [18,26] and assayed at the microarray facility of the Laboratory of Immunopathogenesis and Bioinformatics, SAIC-Frederick, Inc. (Frederick, MD) as previously described [26]. The scanned-image CEL files were processed using a R/Bioconductor package and the robust multichip multi-array average algorithm [26].

Parasite RNA. RNA isolated from synchronized 3D7.G and 3D7.Gdef clones was used to generate fluorescently labeled cDNA as previously described [19] (Table S1) and assayed at NIAID Microarray Research Facility, National Institutes of Health (NIH), Bethesda using the 70-mer oligonucleotide array designed by the DeRisi laboratory (Operon Technologies) [16] as previously described [19].

Patient samples. Patients presenting with P. falciparum at clinics in the Mae Sod district of Tak province in northwestern Thailand were asked if they would be willing to donate blood to study malaria transmission. After completing the informed consent, a venous blood sample was obtained and the plasma was removed by centrifugation and the remaining cell pellet was stored frozen at −80°C. The protocols used to obtain the human samples were reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army.

Whole genome expression profiling. DNA microarrays were long-oligo on glass from Agilent Technologies, Santa Clara, CA designed with custom content. The 44 K spots on each array included probes for 5,254 P. falciparum 3D7 transcript sequences (downloaded from NCBI RefSeq, December 2009). Probes (60-mer) were designed using Agilent eArray software (http://earray.chem.agilent.com/earray/) requesting one probe per target sequence using “best probe” methodology. The array also included probes for 563 human housekeeping-gene transcripts, three spot-replicates each.

Total RNA was isolated from the frozen blood cell pellet using TRIzol (Life Technologies, Grand Island, NY) and purified with the RNaseasy Micro kit (Qiagen, Valencia, CA) and fluorescent labeled using the QuickAmp kit (Agilent). Cy dye labeled DNA from infectious and non-infectious patient samples, paired based on similar asexual and gametocyte parasitemias, were cohybridized on the same slide. Each pair was labeled and hybridized again for dye-swap replicates. Hybridizations were incubated for 17 hr at 65°C on a TECAN HS Pro hybridization station using Agilent hybridization buffers, dried under nitrogen and scanned using a Agilent Microarray Scanner (G2505C) in an ozone-free environment at 5 micron resolution using the extended dynamic range (XDR) mode at a 100:1 ratio. Image analysis was performed using Feature Extraction software (version 10.7.3.1) with protocol GE2_107_Sep09. Background detrend and multiplicative detrend signals were used to account for any spatial effect on the array while no other background subtraction was applied. Dye effect was removed by using linear global normalization followed by non-linear lowess normalization. The processed signals were then subjected to quantile normalization and log transformation in Partek Genomics Suite 6.5 (Partek Inc., St. Louis, MO). Signals from dye swapping samples were then averaged using geometric mean. This provided intensity values for each transcript in individual samples which were comparable across arrays. This intensity-based analysis of dual color microarray data has been shown to give reproducible results and increase sensitivity compared to ratio-based analysis [75]. Unsupervised hierarchical cluster of samples based on a given list of genes was performed in Partek Genomics Suite 6.5 with sample distance determined by Euclidean distance and cluster distance determined by average linkages between all pairs of samples. Genes with expression profiles that correlated with either asexual parasitemia or gametocytemia were assessed by statistical analysis of covariance (ANC OVA). False discovery rate (FDR) was calculated to control for multiple comparisons using the q-value method (FDR q-value) [76].

Chromosome 9 deletion mapping

PCR primers (Table S3) spaced at ~2-kb intervals spanning the region on chromosome 9 between oligonucleotides i14759_1 and i12812_3 (DeRisi laboratory) were used to amplify gDNA isolated from Indochina, 3D7.Gdef, FCR3.Gdef and HB3.Gdef parasite lines.

Parasite transformations

P. falciparum parasites were transformed using established protocols as previously described [14,77]. The complementation and reporter-tagging plasmids used are described below and the primers used for the amplifications are listed in Table S3.

Complementation. The 5’ flanking region, bp −1236 to −1 from the start codon of Pfgdv1, was amplified using primers Pfgdv1.5’FR.-1236.forward and Pfgdv1.5’FR.1.reverse and standard PCR conditions, 35 cycles of 94°C 30 s, 45°C 30 s, 60°C 30 s. The PCR product was digested with SacI and Xbal and ligated into pCBM.BSD [78]. Once the sequence of the resulting plasmid pCBM.BSD.Pfgdv1.5’ was confirmed it was digested with Xbal and ligated to a Xbal-digested PCR product encoding Pfgdv1 (bp 1–1800, chr9:1,377,942–1,379,741) generated using primers Pfgdv1.1.forward.Xbal and Pfgdv1.1800.reverse.Xbal.
Pfgdv1 reporter tagging. Pfgdv1 was tagged with either hemagglutinin (HA) or green fluorescent protein (GFP) to investigate the localization of the protein. Synthetic oligonucleotides encoding the HA epitope tag flanked 5' by a partial XhoI site and 3' by SpeI and a partial BamHI site were phosphorylated with T4 Polynucleotide kinase (New England Biolabs), incubated at 65°C for 10 min in Sequenase buffer (Life Technologies) and annealed by stepwise cooling (42°C for 5 min, 37°C for 5 min, 25°C for 10 min). The annealed oligonucleotides were ligated into XhoI and BamHI digested pCBM.BSD.Pfgdv1.15'. After the insertion was confirmed by sequencing, a PCR product encoding the Pfgdv1 coding region (bp 1 to 1800) flanked by SpeI and BamHI sites generated using primers Pfgdv1.1.forward and Pfgdv1.1800.reverse.BamHI was inserted into SpeI and BamHI digested pCBM.BSD.Pfgdv1.15'.HA to produce pCBM.BSD.Pfgdv1.15'.HA. The entire Pfgdv1.15'.HA. ORF fragment was removed by digestion with SacI and Sall and ligated into Sall and BamHI-digested pDT.Tg23 [79] to generate pDT.Tg23.Pfgdv1.15'.HA. ORF.

To generate GFP-tagged PGDV1, the HA epitope and Pfgdv1 ORF in pDT.Tg23.Pfgdv1.15'.HA. ORF were replaced by a PCR product encoding Pfgdv1.1(1–1797) bp flanked by XhoI and SpeI sites generated using primers Pfgdv1.1.forward.XhoI and Pfgdv1.1797.reverse.SpeI to produce pDT.Tg23.Pfgdv1.15'.ORF. A SpeI and BamHI-digested PCR product containing the GFP coding sequence were set up at 6% hematocrit and sorbitol synchronized 2 hours later to initiate the 3 asexual cycles. The 3 asexual cycles are indicated by the gray box. Data from one of three independent experiments are plotted with the error bars representing the range. (PDF)

Figure S1 Alignment of PGDV1 with homologs in other Plasmodium species. The predicted amino acid sequence of Pfgdv1 (PF11710w) was aligned with homologs identified by BLAST searches of translated sequences on www.PlasmoDB.org, www.ncbi.nlm.nih.gov, and www.sanger.ac.uk/Pathogens/malaria using NPS@: Network Protein Sequence Analysis at www.expasy.org. Shown is the CLUSTALW alignment of PGDV1 (PF11710w) with Plasmodium reichenowi (P332h11.plk), Plasmodium vivax (PVX_086950), Plasmodium knowlesi (PK_073430), and Plasmodium gallinaceum (Pgal0953d04.plk) (37). The Pfgdv1 sequence and exact matches with other species are highlighted in yellow. Identical amino acids (aa) are shown in red, highly similar aa are green and weakly similar aa are blue. The two helix-rich regions predicted by the GOR4 algorithm are underlined. (DOC)

Table S1 Genes differentially expressed in the 3D7.G+ and 3D7.GΔf clonocytes.

| Gene accession numbers | Pfge6, PF14_0744; Pfge2, PF14_0745; Pfge3, PF14_0748; Pfge7, PF14_0736; Pfge8, PF14_0735; msp7-3, PF13_0196; Pf47, PF13_0248; Pf16, PF10_0508; PF14_0520; PF08_0002; PF14_0708; PF14_0745; PF11_0038; PF08_0033; PF14_0010; Pf230, PFB0405w; MCM2, PF14_0177; Ser2A, PF13_015; kahbp, PBFO0100c; aldolase, PF14_0425; seryl tRNA synthetase, PF07_0073; Pf25, PF10_0303; Pf28, PF10_0302. |

Supporting Information

Table S2 Clinical and hematological characteristics of the patient cohort.

*Acknowledgments*

We thank Robert Emnesser, and Christina David for technical support and NIAID intramural editor Brenda Rae Marshall for assistance. Reagents
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P. falciparum gametocytogenesis

Author Contributions

Conceived and designed the experiments: SE BJM YH TF HJ OA HK KK TGM JS XS KCW. Performed the experiments: SE BJM TH TJ OA HK KK AS BC. Analyzed the data: SE BJM TH TJ OA HK KK AS BC: AA TGM JS XS KCW. Contributed reagents/materials/analysis tools: KK JS. Wrote the paper: SE BM TF HX TGM XS KW.
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