Plasmodium's Crossroads: Deciphering the Molecular Pathway That Leads to Malaria Transmission

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Plasmodium’s Crossroads: Deciphering the Molecular Pathway that Leads to Malaria Transmission

A dissertation presented

by

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to

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Plasmodium’s Crossroads: Deciphering the Molecular Pathway that Leads to Malaria Transmission

Abstract

*Plasmodium falciparum* is the causative agent of the most severe form of malaria. Transmission from humans to mosquito vectors is an essential step in this eukaryotic parasite’s life cycle and in the spread of malaria disease, which killed nearly 600,000 people in 2013. I investigated the developmental switch parasites make to the transmission stage or gametocyte, with the goal of identifying molecular mechanisms and environmental triggers of gametocyte formation. In Chapter 2 of this dissertation, I discuss the completion of a genetic mutagenesis screen leading to the discovery of a putative E3 ubiquitin ligase enzyme which is likely a negative regulator of gametocyte formation. Chapter 3 presents findings on population-based regulation of gametocyte production and on parasite-derived microvesicles that transfer between cells and stimulate gametocyte production. In Chapter 4, I and coauthors present a protocol for measuring parasite growth and gametocyte production in response to drugs or other treatments.
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Note: Except as otherwise noted, I. Goldowitz and M. Marti conceived and designed all experiments shown, and I. Goldowitz carried out all experiments shown. Other contributors are indicated in the Author Contributions notes after the appropriate figure legend.
Chapter 1. Introduction
1.1. Background and general summary.

The malignant malaria parasite *Plasmodium falciparum* proliferates within cells of the human body and causes nearly 600,000 deaths each year and serious harm to millions more. Although there are effective treatments, 1.2 billion people remain at high risk of infection\(^1\), and malaria will continue causing deaths, morbidity and economic harm until transmission is effectively contained. During an infection, parasites first invade human liver cells, then move to the bloodstream where repeated asexual replication cycles within red blood cells allow a potentially massive population expansion. This asexual replication cycle is responsible for causing the serious symptoms of malaria, which can include fever, vomiting, anemia, metabolic acidosis, and the frequently fatal complications of cerebral malaria and respiratory distress, and is the chief intended target of all antimalarial drugs.

Malaria does not spread directly from human to human but is vectored by a mosquito in the *Anopheles* genus. A series of specialized parasite stages are required for transmission to and development within the mosquito. The first of these stages is the gametocyte, which forms when asexual blood stages cease replicating and enter the sexual developmental pathway. At maturity the gametocytes are the only stages able to infect a mosquito, where they form gametes. Because of gametocytes’ importance in spreading disease and their biological differences from asexual blood stages, drugs or vaccines that specifically block gametocyte formation or transmissibility would be a valuable addition to the antimalarial arsenal\(^2\). Additionally, a better understanding of how current interventions affect gametocyte formation and transmission, and thus the spread of disease, is required for optimal malaria control.
A strong push against malaria has reduced incidence worldwide by 30% since 2000. Strategies have included antimalarial drug deployment, use of insecticide-treated bed nets, vector control using insecticides, and surveillance\(^1\). The WHO recommends use of artemisinin combination therapies (ACT) in which the fast acting artemisinin derivatives are combined with drugs of different classes. However, the WHO estimates that between 56 and 69 million children with malaria did not receive any ACT in 2013\(^1\). In specific regions and for specific patient populations, other drugs are still used including quinine, chloroquine, sulfadoxine-pyrimethamine, atovacuone-proguanil, and mefloquine\(^3\). Many antimalarial drugs are effective against early gametocytes but have no or much lower activity against gametocytes of Stage III and later\(^4\), and none were developed specifically to kill gametocytes of any stage. Importantly, asexual trophozoites and schizonts adhere to vascular epithelium and sequester in various organs, while immature gametocytes develop in sequestration mainly in the bone marrow extravascular space\(^5\) and re-enter peripheral circulation when they reach maturity. This could potentially make developing gametocytes less accessible to drugs once they sequester.

Several drug classes have been reported to stimulate the formation of gametocytes in patients or in vitro (discussed in Chapter 3). It is possible that this phenomenon could lead to fully or partially drug resistant parasites spreading preferentially from treated patients, as has been observed in rodent infection experiments with the related *Plasmodium chabaudi*\(^6\). Also, as gametocytes are the sexually reproducing stage these escapees from treatment could form gametes and allow several full or partial drug resistance markers to recombine. Multi-drug resistant *P. falciparum* parasites are now transmitting in regions of Southeast Asia\(^1\). These observations raise the specter of multi-drug resistant parasites spreading to additional regions,
which could lead to increases in death rates as occurred when chloroquine resistance spread between the 1950s and the 1990s. For example, the rise of parasite chloroquine resistance and treatment failure in the late 1980s probably were responsible for the 2.5fold to 11fold increases in malaria deaths which occurred in villages in Senegal\textsuperscript{7}.

Local elimination and global eradication have once again been identified as goals by the public health and malaria research communities, and better strategies for controlling transmission and transmission stages may be key for meeting these goals\textsuperscript{8}. To achieve this, researchers must make progress in understanding the biology of gametocytes and the influences on their formation, survival and transmissibility.

Like most members of the exclusively parasitic Apicomplexan phylum, \textit{P. falciparum} possesses a complex life cycle. This parasite makes dramatic changes to its morphology, gene expression and physiology in order to adapt to the multiple cell types and microenvironments it inhabits within the bodies of two species. Progression through the life cycle is generally very regimented in \textit{P. falciparum}, and is controlled by transcriptional cascades which are shepherded along by the ApiAP2 family of transcription factors\textsuperscript{9}. Each life cycle transition is predetermined or 100\% triggered by a transition between species, except for the transition between asexual blood stage cycling and sexual development as a gametocyte. In every intraerythrocytic round, parasites may decide to 1. continue proliferating asexually within erythrocytes or 2. irreversibly stop replicating and enter the 8-12 day sexual development pathway. The latter is termed sexual conversion.

The aim of my dissertation research has been to discover cellular mechanisms of sexual conversion in \textit{Plasmodium falciparum} and to add to our understanding of how parasite
populations and individual cells regulate this critical life cycle decision. I hypothesized that the sexual conversion decision in each parasite relies on a cellular pathway which is either induced or not induced through external and internal factors, leading to production of either sexual or asexual offspring. Up until five years ago, no mechanistic component of sexual conversion had been identified. Potential environmental and drug triggers of the switch had been reported, and genetic defects leading to the loss of gametocyte development or the death of most early gametocytes had been described, but how these act within cells to trigger or block conversion remained unknown.

In the course of my research, I discovered that a putative E3 ubiquitin ligase enzyme acts as a negative regulator of gametocyte conversion and is required for normal cell cycle progression (Chapter 2). I collaborated with Dr. Pierre-Yves Mantel and others to show that microvesicles released by parasite-infected RBCs into the culture medium carry information from cell to cell and stimulate gametocyte conversion (Chapter 3). In collaboration with Dr. Nicolas Brancucci I helped improve a protocol for evaluating the effect of drugs on gametocyte conversion and early development by incorporating my findings about cell-cell communication and strain differences into a previously reported method (Chapter 4). These finding will contribute to the parasitology field’s understanding of the logic and mechanisms of cell fate determination at this crossroads of proliferation and transmission.

1.2. Evolution of sexual development in the Apicomplexa.

An evolutionary perspective can help us make predictions about how the asexual-to-sexual transition could be regulated in P. falciparum. Malaria parasites are members of the
Apicomplexa, a eukaryotic phylum of exclusively parasitic single-celled microbes. Most Apicomplexans possess a complex, multi-stage life cycle in which only one stage is sexual and the rest are asexual stages specialized for different environments within their hosts and/or vectors. Compared to its relatives, *P. falciparum* shows especially low gametocyte production per round together with a potentially massive expansion of the asexual population during blood cycling (Figure 1). Other *Plasmodium* species, including parasites infecting lizards and birds, show much higher sexual conversion per round and limited asexual expansion. For example, a study of *Plasmodium mexicanum* infections of wild lizards showed that the ratio of gametocytes to asexual parasites increased rapidly after initial transmission until it dominated each infection, and in stable infections, gametocytes accounted for between 50% and 100% of visible parasites in all lizards sampled.\(^{10}\)

In many Apicomplexans there is no similar expansion of the life cycle stage immediately preceding the gametocyte; instead, gametocytes are produced after just one or a small, predetermined number of asexual replication rounds (Figure 1). Parasites of *Hemoproteus* and *Hepatocystis*, both members of the Hemospororina suborder along with *Plasmodium*, transform into gametocytes immediately after their first erythrocytic cycle (reviewed Smith et al.\(^ {11}\)). *Eimeria tenella*, a parasite of domestic chickens belonging to the coccidian subclass of Apicomplexans, undergoes two or three distinct rounds of asexual replication and reinvasion in the host gut cells. All cells from either the second or third round of schizogony then form gamonts (the precursor to gametes), depending on the strain (reviewed Smith et al.\(^ {11}\)). Another coccidian, *Cryptosporidium parvum*, proceeds through one or a few Type I merogony cycles and a single Type II merogony cycle before forming gamonts.\(^ {12}\)
Figure 1. Sexual development behavior across the Hemospororida, an Apicomplexan group.

Left: A partial phylogeny of the Hemospororida with vectors and hosts. Image reprinted with permission from Martinsen et al. (2008)13. Right: Progression to sexual development in the pictured clades. *Hemoproteus* parasites and most other members of the Hemospororida begin sexual development immediately upon invading erythrocytes. *Plasmodium* species, in contrast, undergo an indefinite number of schizogonic rounds in human RBCs, with a small (*P. falciparum*) or large (many avian and reptilian *Plasmodium* parasites) proportion of cells exiting the cycle after each round to develop sexually. Smith et al. (2002)11 argue based on phylogenetic evidence that proceeding directly to sexual development is likely the ancestral state in Apicomplexans and that the asexual population expansion of schizogony evolved later.
Figure 1. (Continued)
Smith et al. (2002)\textsuperscript{11} argue that the likely ancestral state in Apicomplexans was to proceed immediately to gametocyte development after a single round of the previous asexual stage. For blood parasites, that correlates to gametocyte development in the first erythrocytic invasion, while asexual schizogony in erythrocytes would be a derived state that arose later\textsuperscript{11}. This suggests that repressive mechanisms may have evolved in species that use erythrocytic schizogony to delay sexual development that would otherwise occur as a default.

Based on this likely evolutionary history I hypothesized that in \textit{P. falciparum}, negative regulatory mechanisms are in place that prevent gametocyte conversion and allow asexual cycling. Thus, in investigating the mechanisms of gametocyte conversion, it could be productive to look for genetic changes that can increase gametocyte production in addition to those that can ablate it (Chapter 2). Interestingly, genetically stable \textit{Eimeria tenella} mutants (known as “precocious” mutants) have been found that form gamonts directly from first stage schizonts, instead of from second and third stage schizonts as normal strains do\textsuperscript{14}. These strains could potentially have a defect in a regulatory mechanism that represses gamont development during first and second stage schizogony. Precocious \textit{Eimeria} lines never produce the pathogenic asexual population expansion that normal strains do, and they are therefore in use as live attenuated vaccines to prevent \textit{E. tenella} infection in poultry\textsuperscript{15}. This also highlights the idea that a faster switch to sexual development can restrain the pathogenicity of an infection.

Conversely, it is likely that the especially large expansion in \textit{P. falciparum} asexual schizogony is one cause of the high pathogenicity of \textit{P. falciparum} infection; this is partially due to the lower sexual conversion rate in this species than in many of its relatives.
1.3. Recently discovered and hypothesized mechanisms controlling sexual conversion

The sexual conversion decision in *P. falciparum* is thought to occur during the previous blood stage asexual cycle, leading to sexually committed schizonts which reinvade to form young gametocytes\textsuperscript{16}. In the malaria intraerythrocytic cycle, asexual cells (ring and trophozoite stages) increase in size while remodeling their host RBC and consuming its contents; they also consume nutrients imported from the bloodstream. Multiple rounds of DNA replication in the trophozoite lead to a multinucleate cell called a schizont, which delays cytokinesis until approximately 48 hours post invasion when up to 32 invasive merozoites separate and burst from the host RBC. In this model, at some point before formation of late schizonts, each cell decides whether to form sexual or asexual merozoite offspring. A schizont destined to produce sexual offspring already shows transcriptional signatures of its decision\textsuperscript{17} and is referred to as a committed schizont.

The conversion rate, or the proportion of parasites undergoing sexual conversion in each cell cycle, has been reported to vary according to the parasite’s environment (see Chapters 3 and 4), genetic background (see Chapter 2), and epigenetic status (see below and Chapter 2). There is additionally a stochastic aspect to the conversion decision, evidenced by the fact that a small but nonzero proportion of a clonal parasite population typically convert during each round. Thus, parasites with the same genetics, exposed to the same environmental factors, and existing in the same population still differ in their conversion outcome.

Recent discoveries demonstrate that genes controlling early gametocyte development are indeed under epigenetic repression in most cells, preventing sexual conversion and allowing
expansion of the asexual population. Two teams showed that the gene Pfl1085w (Pf3D7_1222600), and the corresponding gene PBANKA_143750, are essential for gametocyte production in both P. falciparum and the rodent malaria parasite P. berghei respectively. These genes encode a member of the ApiAP2 family of transcription factors which regulate progression through the malaria life cycle by controlling transcriptional cascades. The protein, renamed AP2-G, acts as a transcriptional master switch for sexual stage activation in both species. In P. falciparum, AP2-G binds to a motif in the promoters of early gametocyte genes including Pf14_0744 (Pf3D7_1477300), which is expressed as early as the committed schizont stage, and can also activate its own transcription in a positive feedback loop.

I played a small role in a project to study PfHDA2, a class 2 histone deacetylase which is part of the histone code writing system of epigenetic regulators. We found that deficiency of HDA2 in a knockdown strain of P. falciparum leads to increased sexual conversion and increased gametocytemia in a gametocyte induction assay (Figure 2a). PfHDA2 helps recruit heterochromatin protein 1 (PfHP1) to specific target regions of DNA by removing acetyl marks from histones, which leads to silencing of genes in the targeted regions; these regions include genes involved in early gametocyte development. In another recent publication, heterochromatin protein 1 (HP1), a phylogenetically widespread and conserved protein, was shown to promote heterochromatin formation and silence large regions of the P. falciparum genome when it binds non-acetylated, methylated histones (specifically H3K9Me3). Removal of HP1 in knockdown parasites was likewise shown to greatly increase gametocyte production and block asexual cycling. The gene encoding AP2-G lies in one of the regions marked by HP1, as does the committed schizont marker Pf14_0744. Thus, HDA2 and HP1 are both negative
Figure 2. (A) A knockdown of PfHda2 protein leads to increased gametocyte production. A representative experiment (unpublished but used as preliminary data for Coleman et al. 2014, Cell Host & Microbe\textsuperscript{20}) is shown. Two knockdown clones of 3D7/HDA2-DD and wild type parasites of reference strain 3D7 were assayed for gametocyte production and total parasite proliferation according to the Gametocyte Induction Assay shown in Figure 3. Parasites were either maintained ON 500 nM of Shield-1 or washed 4x and taken OFF Shield-1 either one or two cycles before the assay start. In this inducible knockdown line, removal of Shield-1 leads to destruction of the DD-tagged HDA2 protein. Means of three or four technical replicates are shown. (B) Negative and positive regulation of gametocytogenesis by proteins HP1 and HDA2 along with hypothesized external and internal inputs. Sexual conversion is hypothesized to involve a cellular pathway that includes cellular stress response mechanisms, signaling molecules, and epigenetic control mechanisms which integrate information from within and outside the cell. HDA2 removes acetyl marks from histones and HP1 binds methyl marks on histones; both help impose silencing on targeted genomic regions, leading to silencing of the genes encoding gametocytogenesis transcription factor AP2-G and early gametocyte proteins Pf14_0744 and Pf14_0748. Pro-conversion signaling is predicted to oppose the activity of HDA2 and HP1; these hypothesized interactions are depicted with dotted lines. Author contributions: For (A), B. Coleman, K. Skillman and coauthors designed and constructed the HDA2-DD knockdown lines. K. Skillman, I. Goldowitz, M. Duraisingh, and M. Marti conceived and designed the experiment shown. I Goldowitz carried out the experiment under the supervision of M. Marti.
Figure 2. (Continued)
regulators of sexual conversion which cooperate to impose epigenetic silencing on gametocyte genes in most cells in a population (Model, Figure 2b). HDA2 and HP1 were additionally found to silence non-dominant var genes, members of a polymorphic family involved in parasite sequestration and immune evasion of which only one member is expressed at a time\textsuperscript{20,21}.

Gametocytes can be either male or female and, upon uptake by a mosquito, produce eight flagellated microgametes (male) or a single macrogamete (female). Sex determination is a second aspect of gametocyte development that is probably influenced by the environment, as sex ratios change over the course of a human infection. The optimal sex ratio for transmission may depend on gametocyte density\textsuperscript{22}. It is not known whether sex determination occurs concurrently with the decision to commit to asexual or sexual development, or later during gametocyte development; if the former, aspects of the sex determination mechanism could be shared with the conversion mechanism, and it is possible that some of the stimuli that alter conversion rates could affect only males or only females.

**1.5. Gametocyte detection and challenges.**

Measurement of gametocytemia and sexual conversion rate can be challenging for several reasons: gametocytes are a relatively low-abundance cell type and young gametocytes are morphologically indistinguishable from asexual parasites until they are approximately 55-60 hours old. However, several molecular markers can distinguish gametocytes when they are 60-80 hours old; these include Pfg27 (Pf3D7_1302100), Pfs16 (Pf3D7_0406200), and Pf10_0164 (Pf3D7_1016900). Several genes are expressed and can distinguish sexual cells as early as the
sexually committed schizont; these include \textit{Pf14\_0744} (Pf3D7\_1477300) and \textit{Pf14\_0748} (Pf3D7\_1477700)\textsuperscript{17}. Throughout this dissertation I use variations of a Gametocyte Induction Assay (GIA) described in Chapter 2.2 and in Figure 3, which is modified from a protocol described in Buchholz et al. (2011)\textsuperscript{23}. Parasitemia (the percentage of erythrocytes which are infected with a parasite) and gametocytemia (the percentage of erythrocytes infected with a gametocyte) are both measured and are used to calculate the conversion rate from a single invasion round, defined as gametocytemia/parasitemia x100.

In the GIA, parasitemia is detected using DNA dye labeling of infected erythrocytes followed by flow cytometric quantification. Several gametocyte detection methods are used, depending on the requirements of the experiment. These include immunofluorescence labeling with antibodies to the gametocyte-specific protein Pfg27 followed by flow cytometric quantification (Chapter 2), light microscopy with morphological counting of 64-74 hour old early Stage II gametocytes, when gametocytes stemming from a single invasion round are unambiguously distinguishable (Chapters 2 and 3), and flow cytometric detection of the transgenic strains 3D7/0164-GFP, 3D7/744-GFP, and Pf2004/0164-TandemTomato which express fluorescent proteins specifically during gametocyte stages (Chapters 3 and 4). After evaluating the performance of antibodies against Pfs16 and Pfg27 by comparing morphology, fluorescence microscopy, and flow cytometry results, an antibody against Pfg27 (kind gift of Dr. Kim Williamson) was chosen. Pfg27 is a highly abundant protein that binds RNA in gametocytes\textsuperscript{24}, and provided lower background on asexual cells at a concentration that brightly stains gametocytes.
Chapter 2: A putative malaria E3 ubiquitin ligase represses gametocyte conversion and maintains asexual cycling in *Plasmodium falciparum*.
Abstract

To identify genes potentially involved in the formation of the *Plasmodium falciparum* gametocyte, or human-to-mosquito transmission stage, I performed a small-scale genetic screen of a transposon-mutagenized library constructed in a gametocyte producing parasite line (Balu et al 2009, 2010). I identified two disrupted lines which differentiate into gametocytes at dramatically increased rates and two which form gametocytes at unusually low rates relative to their wild type parent. I focus here on one of these lines, in which a disruption of its promoter leads to reduced expression of gene *Pf13_0164* (PF3D7_1330500). This previously unstudied gene codes for a putative RING type E3 ubiquitin ligase. Further characterization of the mutant reveals that, in addition to constitutively producing high levels of gametocytes, it displays multiple phenotypes which have been linked in recent reports with sexual commitment. Construction of an independent knockdown of *Pf13_0164* protein in a wild type strain illuminates a link between gametocyte conversion and cell cycle regulation. Based on these findings it is likely that the *P. falciparum* gene *Pf13_0164* codes for an active RING-type E3 ubiquitin ligase, which acts as a repressor of gametocyte development upstream of the gametocytogenesis transcription factor AP2-G. It is therefore likely to be essential for asexual proliferation.
2.1. Introduction

*Plasmodium falciparum* is the eukaryotic parasite responsible for the most dangerous form of malaria and for killing over 500,000 people each year. A gap in malaria professionals’ knowledge has been how best to constrain transmission of this deadly parasite from patients to the mosquito vector, which allows the infected mosquito to eventually transmit back to humans. Less is understood about the stages of the parasite life cycle that transmit to and those that develop within the mosquito and these stages have traditionally been more difficult to study. The rising spread of drug resistant parasites as well as insecticide resistant mosquito vectors\(^1\) increases the urgency of understanding transmission stages and how they form.

The process of transmission begins when parasites proliferating asexually in the human bloodstream cease replicating and develop as a sexual form called the gametocyte. When a mosquito bites an infected person, it can take up mature gametocytes present in the blood meal and become infected. Within the mosquito the parasites undergo sexual reproduction and proceed through several life cycle stages, then develop into the sporozoite form which can infect humans. The pathway controlling the switch to gametocyte development, known as sexual conversion, is poorly understood, but once activated in a subset of the population, these parasites irreversibly leave the 48-hour asexual proliferation cycle in favor of the 8-12 day long sexual development pathway.

Only a few genes directly involved in sexual conversion have been identified. Permanent genetic losses of gametocytogenesis have arisen in long-term cultured parasites; several of these have been studied with the goal of identifying the defect. A gametocyte-deficient clone of reference strain 3D7 was used to identify the gene *GDV1* as being essential for production of
early gametocytes. GDV1 is expressed specifically in committed schizonts and gametocytes but its precise function is still unknown. Another gametocyte-deficient derivative of 3D7, known as F12, was used along with other evidence to show that a transcription factor, AP2-G, acts as a master switch for sexual conversion by activating the earliest known genes in gametocyte development. Additionally, two epigenetic regulatory proteins, HP1 and the class II histone deacetylase HDA2, were shown to be negative regulators of conversion, and conditional knockdowns of each of these led to increased gametocyte conversion.

Of the ~5,300 predicted genes in the P. falciparum genome, the majority are of unknown function, and as of 2002 almost 60% did not have even a provisional function assigned as they were not sufficiently similar to proteins from any better-studied organism. In other systems, genetic mutagenesis screens have been a fruitful way to identify the genes required for functions and pathways of interest; the initial mutagenesis of the genome can be accomplished by agents including DNA-damaging chemicals, radiation, retroviruses, or transposons.

Transposon mutagenesis has become popular in Plasmodium genetic screens aiming to assign functions to unknown genes. Sakamoto et al (2005) performed a transposon mutagenesis screen in P. berghei using a shuttle strategy to create pools of knockout parasites and identify genes essential at different points in the parasite life cycle. Balu et al. (2005) showed that random insertional mutagenesis of P. falciparum is possible by transforming cells with both a transposase and the piggyBAC lepidopteran transposable element. In this technique, which has been extensively used in mutagenizing invertebrates, the transposon randomly inserts into and disrupts genes or regulatory regions. Random insertional
mutagenesis with the piggyBAC transposon is particularly advantageous for malaria genetic screens because its TTAA insertion site is a good match for AT-rich *Plasmodium* genomes, and because the location and uniqueness of the transposon insertion can be determined by inverse PCR or by transposon-directed insertion site sequencing (TRADIS\textsuperscript{30}).

Ikadai et al. (2013)\textsuperscript{31} used the piggyBAC transposon to mutagenize a *P. falciparum* line which expresses GFP in mature gametocyte stages, and screened 189 clones to identify those that cannot produce mature gametocytes. They identified 16 unique genes whose disruption led to loss of gametocyte conversion or development. Four out of five mutants tested showed successful rescue of the phenotype when a normal copy of the gene was supplied on an episomal plasmid. Thus, despite the relatively small size of the library, covering at most 3.5\% of the genome, the researchers were able to identify several genes putatively involved in gametocytogenesis, probably because conversion and gametocyte development are complex pathways which require many cellular processes.

The Adams Lab piggyBAC mutagenized library used in this screen was constructed in the *P. falciparum* strain NF54, which is probably of West African origin and is the parent of the well-characterized reference strain 3D7\textsuperscript{32}. At the time of the screen, the library included 126 unique mutants each of which had been determined to contain a single transposon insertion by TRADIS sequencing; these mutants were assayed for defects in asexual blood stage proliferation and 50\% showed some level of impairment\textsuperscript{33,34}. This library has since been expanded to >1200 disrupted mutants, for a genome coverage of \textasciitilde 20\%.

**Results**
2.2. Development of an assay able to detect differences in conversion behavior among parasite lines.

For use in screening this library, I developed an assay (Figure 3a, Gametocyte Induction Assay, “GIA”) modified from that reported in Buchholz et al. (2011), to measure the conversion rate of any *P. falciparum* parasite line under a standard set of conditions. In the assay (Figure 3a), tightly synchronized early trophozoites of each line are seeded in 96 well plates, and gametocyte production is induced using parasite-conditioned medium produced by incubation with parasites of the same strain. After the parasites reinvade, at the appropriate time points parasitemia (%P) is quantified via flow cytometry with DNA dye staining of all parasite-infected RBCs. Gametocytemia (%G) is read via flow cytometry with immunofluorescence staining of gametocytes using the abundant gametocyte-specific marker Pfg27 (see Methods); alternatively, the induced gametocytes are counted by light microscopy when they are 64-72 hours old, when they are easily distinguishable from asexual cells and from gametocytes stemming from other reinvasion rounds. By contrast to the previously reported gametocytogenesis screen by Ikadai and coauthors, this GIA uses an earlier readout and measurement of both parasitemia and gametocytemia deriving from a single reinvasion round, which allows calculation of the percent conversion as: (%G)/(%P) *100= %C. The screen is thus designed to discover mutations that impair very early gametocyte development or that either block or activate the conversion pathway itself.
Figure 3. Development and use of a Gametocyte Induction Assay (GIA) to quantify conversion rate in any strain under a set of standard conditions. (A) Work flow of the GIA. (B) A panel of *P. falciparum* strains exhibits different parasitemia (left) and gametocytemia (right) when treated in identical culture conditions. (C) The percent conversion is calculated as % gametocytemia/% parasitemia x100. After being cultured at 4% hematocrit for 3 cycles and loosely synchronized, strains are tightly synchronized by MACS magnetic purification followed by sorbitol synchronization four hours later. At 20-24 hours old, the cells are diluted to 1% parasitemia and 2% hematocrit using culture medium consisting of 50% fresh and 50% conditioned medium produced by incubation on the same strain, and seeded in 96 well plates. Approximately 48 hours later, at 24 hpi, the medium is exchanged, a sample from each well is stained with a DNA dye, and % parasitemia is quantified by counting 50,000 events in the appropriate channels on a flow cytometer (see Methods). Several techniques may be used for detection of gametocytes; in this experiment and in Figure 4b, immunofluorescence staining and flow cytometry were used. At approximately 64 hpi, a sample from each well was fixed and immunofluorescence staining was performed using antibody to Pfg27 (see Methods). Percent gametocytemia was quantified by counting 200,000 events on the FITC channel of a flow cytometer. For both % parasitemia and % gametocytemia readouts, control uninfected RBC wells incubated in the plate were used to subtract flow cytometry background. It is also possible to add a drug or other treatment at any point prior to parasitemia readout.
Figure 3. (Continued)
As a pilot for the GIA, I measured % gametocytemia and % parasitemia and calculated conversion rates of five *P. falciparum* strains (Figure 3b, c). This revealed a surprising level of variability in conversion rates even among cultured wild type strains when treated under identical environmental conditions. This could reflect differences between strains in a baseline conversion rate or in environmental susceptibility to the conditions encountered in the assay.

### 2.3. Screening, initial results and hit selection.

Seventy-nine piggyBAC-disrupted lines were assayed using the GIA for % parasitemia and % gametocytemia (Figure 4a, b), which should provide coverage of approximately 1.5% of *P. falciparum* genes. Controls in each plate included the library’s parent strain NF54, the gametocyte-deficient strain F12 as a negative control, and uninfected RBCs. The recently field derived strain Pf2004"""" was also included in each plate as a positive control for gametocyte induction, as it attains high conversion rates under the conditions of the assay (Figure 3b).

Conversion rates (Figure 4c) were calculated from the measured parasitemia and gametocytemia values. The mean conversion rate of 79 library strains was 4.04%. I set upper and lower thresholds at a level which would allow selection of ten hits, leading to selection of four disrupted lines with unusually high and six with unusually low conversion rates.

The ten initial “hit” strains were retested (Figure 4d) using the GIA in three additional biological replicates, including at least one additional cryopreserved stock of the same original library clone. In retesting, gametocytemia readouts were obtained by light microscopic morphology counting, which provided confirmation by a second method. Analysis of the
Figure 4. Screen of a transposon-mutagenized parasite library and hit confirmation. (A) Initial results for % parasitemia, (B) % gametocytemia, (C) conversion rate. Each strain was tested using the Gametocyte Induction Assay shown in Figure 3. As controls, several wells of parental NF54 and of uninfected RBC were included in each plate along with several wells of the strain Pf2004, which produces high conversion rates under the conditions of the assay, and the gametocyte deficient strain F12. Graphs show mean and sem of five technical replicates, or for the controls, mean and sem of at least three biological replicates. (D) Conversion rates upon retesting of 10 hit strains. Three independent biological replicates of each mutant were
Figure 4 (continued)

synchronized and tested in the Gametocyte Induction Assay, along with two normal strains as controls. At least one separate original frozen stock from the library construction was included for each mutant to reduce the possibility of interference from new mutations arising during culturing. Because of flow cytometry problems, the gametocytemia readout was performed by counting gametocytes per 20,000 RBCs on Diff-Quik stained slides instead of by flow cytometry. The graph shows means and sem of three biological replicates, each obtained by taking the mean of four or five technical replicates.

Author contributions: The initial screen was conducted by I. Goldowitz in the laboratory of Dr. John Adams at the University of South Florida and was supervised by M. Marti and J. Adams. Dr. N. Singh thawed and cultured the library strains until they were ready for synchronization.
retesting results (Figure 4d and Table 1) confirmed that conversion rates of the two high gametocyte producer lines, PB-60 and PB-65, were highly significantly different from the full library by ANOVA (Tukey’s post test) and were also significantly different by the Mann-Whitney U, a nonparametric test which is typically more stringent. Two low gametocyte producing lines, PB-5 and PB-30, reached significance by Mann-Whitney U but not by ANOVA. Taking these four strains as hits provides a hit rate of ~5%, which is comparable to the 11.6% hit rate found by Ikadai et al. considering that the GIA does not identify deficiencies in mid-late gametocyte development.

**Hit strain characteristics.** The two confirmed high-gametocyte producing hits have transposon insertions in intergenic regions, while both confirmed low gametocyte hits have insertions within coding sequences (Figure 5a). In all cases the potentially affected genes are of unknown function, and only two contain identifiable predicted domains. In the mutant with the strongest phenotype, PB-60, the transposon insertion is 10 bp upstream of the *Pf13_0164* (Pf3D7_1330500) start site and 3kb upstream of the *MAL13P1.163* (Pf3D7_1330400) start site. Expression of *Pf13_0164* is thus highly likely to be impacted by the disruption of its promoter region, while *MAL13P1.163* could potentially be impacted as well. Interestingly, the predicted protein for Pf13_0164 (Figure 5b) contains a RING domain, which is the catalytic domain of one of the two types of E3 ubiquitin ligases, along with CTLH and LisH domains which are also characteristic of E3 ubiquitin ligases (Figure 5b).
Table 1. Hit Strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean percent conversion</th>
<th>ANOVA/Tukeys P value; significance</th>
<th>Mann-Whitney U U; P value; significance</th>
<th>Potentially disrupted gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-60</td>
<td>19.33%</td>
<td>&gt;0.0001; ***</td>
<td>0; 0.0009; **</td>
<td>Pf13_0164 (Pf3D7_1330500)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MAL13P1.163 (Pf3D7_1133400)</td>
</tr>
<tr>
<td>PB-65</td>
<td>14.10%</td>
<td>&gt;0.0001; ***</td>
<td>0; 0.0009; **</td>
<td>PFF0295c (Pf3D7_0606000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PFF0300w (Pf3D7_0606100)</td>
</tr>
<tr>
<td>PB-30</td>
<td>0.51%</td>
<td>ns</td>
<td>2; 0.001; **</td>
<td>Pf13_0243 (Pf3D7_1345800)</td>
</tr>
<tr>
<td>PB-5</td>
<td>1.11%</td>
<td>ns</td>
<td>5; 0.0058; *</td>
<td>Pf11_0431 (Pf3D7_1122900)</td>
</tr>
</tbody>
</table>

Table 1. Hit strain characteristics and statistical analysis. For this analysis, first, the five technical replicates (wells in a 96 well plate) were averaged to provide a mean percent conversion for each biological replicate. Then, one-way ANOVA with Tukey’s post test and the nonparametric Mann-Whitney U test were performed using GraphPad Prism version 5.0c for the mutants against the screening results of the full library. Both high gametocyte-producing strains reach strong significance in both tests, but it is more difficult to clearly distinguish the low gametocyte producers from the full library, likely because of variability in conversion rates among technical replicates.
Table 1. Summary of transposon insertion strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PB-30</th>
<th>PB-5</th>
<th>PB-65</th>
<th>PB-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % conversion</td>
<td>0.51</td>
<td>1.1</td>
<td>14.1</td>
<td>19.3</td>
</tr>
<tr>
<td>Asexual growth phenotype</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Impaired (50% of parent)</td>
</tr>
<tr>
<td>Insertion Location</td>
<td>Coding sequence</td>
<td>Coding sequence</td>
<td>Intergenic</td>
<td>Intergenic</td>
</tr>
<tr>
<td>Gene 1 name</td>
<td>Pf13_0243</td>
<td>Pf11_0431</td>
<td>261bp 5' of PFF0295c</td>
<td>10bp 5' of Pf13_0164</td>
</tr>
<tr>
<td>Gene 1 description</td>
<td>Conserved protein</td>
<td>Membr. skeletal protein</td>
<td>Conserved protein</td>
<td>Conserved protein</td>
</tr>
<tr>
<td>Gene 1 peak expression</td>
<td>late gameteocyte</td>
<td>ring and trophozoite</td>
<td>ring</td>
<td>late troph., late gam.</td>
</tr>
<tr>
<td>Gene 1 predicted domains</td>
<td>none</td>
<td>PM</td>
<td>none</td>
<td>RING, LisH, CTLH</td>
</tr>
<tr>
<td>Gene 2 name</td>
<td>none</td>
<td>IMC</td>
<td>3400bp 5' of PFF0300w</td>
<td>3625bp 5' of MAL13P1.163</td>
</tr>
<tr>
<td>Gene 2 description</td>
<td>RNA binding protein</td>
<td>ER lumen retaining receptor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. For each of the four confirmed hit strains, (A) genomic loci disrupted by the transposon insertion, confirmed by TRADIS sequencing, and predicted proteins whose coding sequences or promoters are potentially disrupted by the transposon insertion. Domain predictions are from an InterProScan sequence search. Expression timing predictions are from Le Roch et al. (2003) and Young et al. (2005). All of the probable disrupted genes are of unknown function, but one gene, Pf11_0431, belongs to a structural protein class important in development of other Apicomplexan transmission stages. (B) Predicted domains in Pf13_0164. RING domains are the catalytic domain of one of the two classes of E3 ubiquitin ligases, and CTLH and LisH are also typical of many RING-type E3 ligases.
To further characterize the confirmed hits, I examined their asexual cells and gametocytes by light microscopy on Diff-Quik stained slides. None of the strains had obvious morphological defects, and all produced mature male and female gametocytes.

2.4. Quantitative RT-PCR analysis of hit strain PB-60.

I designed a quantitative RT-PCR study to a) determine how the transposon insertion affects expression of the genes flanking the insertion site in mutant PB-60, and b) understand the timing and effects of this mutant’s defect relative to activation of known early gametocyte genes. For this purpose, I assayed expression of the potentially disrupted genes and of three early gametocyte genes in PB-60, in NF54, and in F12 (Figure 6a). F12 is a strain derived from the reference strain 3D7 which cannot produce gametocytes due to a mutation in transcription factor AP2-G, which initiates sexual development in *P. falciparum* \(^\text{18}\). AP2-G binding motifs are found in the promoters of several early gametocyte genes, including *Pf14_0744* (*Pf3D7_1477300*). This gene and the early gametocyte gene *Pf14_0748* (*Pf3D7_1477700*) are expressed as early as the committed schizont stage, peak at early Stage II, and rapidly disappear by Stage III of gametocytogenesis\(^\text{17}\). AP2-G also stimulates its own transcription in a positive feedback loop. The relative expression of these three genes in PB-60, NF54, and F12 should aid in understanding whether PB-60’s defect occurs before, concurrent with, or after the activity of these genes.

*Assay design.* Several primer pairs were designed for each potentially disrupted gene and for the early gametocyte markers *PFL1085w* (AP2-G), *Pf14_0744* and *Pf14_0748*. Primers
were also designed for PF08_0085 (UCE), a constitutive housekeeping gene used as an endogenous reference gene. For each primer pair, standard curves were generated with genomic DNA of known concentration to determine efficiencies and limits of detection (Table 2) and the best pair for each gene was chosen and validated (see Methods).

Sample collection, PCR and data analysis. RNA collection timecourses (Figure 6a) were performed in PB-60, NF54, and F12 after tightly synchronizing early ring parasites and seeding them at 1% parasitemia in flasks. Sexual conversion was induced at 11 hours post invasion (hpi) of these rings with addition of 50% conditioned medium. RNA samples were collected at six timepoints spanning asexual and early sexual development. After RNA extraction and complementary DNA synthesis, PCR reactions were run using a ViiA 7 real-time PCR system and analyzed using the ΔΔCT method with correction for primer efficiency\textsuperscript{39} (see Methods).

Cycle progression and gametocyte production during timecourse experiments. In the wild type NF54, the proportion of gametocytes appearing in the first cycle is quite low, therefore the first timepoint (10 hpi) almost entirely consists of asexual rings. The second and third timepoint include both sexually committing and asexually committing trophozoites. The final three timepoints contain both asexual cells and young gametocytes (depicted in Figure 6a). F12 asexual cells progressed in a similar manner to those of NF54, but no gametocytes were produced. By contrast, in the PB-60 cultures, gametocytes stemming from previous cell cycles were visible on slides even at the first timepoint, indicating constitutively high gametocyte production. Interestingly, during the timecourse experiments I noted that PB-60 appears to have a cell cycle delay of approximately 8 hours as estimated by light microscopy. This phenotype is discussed in Sections 2.5 and 2.6. This cycle delay affects the interpretation of the
Table 2. Primers selected for quantitative RT-PCR assay. One malaria genome is approximately 0.025 pg. Primer characteristics were evaluated as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Identity</th>
<th>Amplicon Length</th>
<th>Efficiency</th>
<th>Tested Limit of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCE</td>
<td>Endogenous reference gene</td>
<td></td>
<td>100%</td>
<td>.043 pg</td>
</tr>
<tr>
<td>Pf13_0164</td>
<td>Likely disrupted in PB-60</td>
<td>194 bp</td>
<td>88.9%</td>
<td>.033 pg</td>
</tr>
<tr>
<td>PFL1085w</td>
<td>AP2-G, committed schizont marker</td>
<td>118 bp</td>
<td>98.92%</td>
<td>.047 pg</td>
</tr>
<tr>
<td>Pf14_0744</td>
<td>Committed schizont and early gametocyte marker</td>
<td></td>
<td>100%</td>
<td>.043 pg</td>
</tr>
<tr>
<td>Pf14_0748</td>
<td>Early gametocyte marker</td>
<td></td>
<td>97.6%</td>
<td>.043 pg</td>
</tr>
<tr>
<td>MAL13P1.163</td>
<td>Potentially disrupted in PB-60</td>
<td>188 bp</td>
<td>81.6%</td>
<td>.043 pg</td>
</tr>
</tbody>
</table>

**Author contributions**: Primers for Pf14_0744, Pf14_0748, and UCE were designed by Dr. R. Joice.
FIGURE 6. Quantitative RT-PCR study design and results. (A) Study design. Tightly synchronized parasite cells are seeded in flasks at 1% parasitemia and 4% hematocrit in fresh culture medium. To induce gametocytogenesis in a subset of cells, conditioned medium (produced on 2% parasitemia cultures of the same strain from hours 30 to 44 of the life cycle) is added during the first cell cycle at 11 hours post invasion (indicated by the blue arrow) to halve the hematocrit to 2%. Expression of the potentially disrupted genes and of three early gametocytogenesis genes is measured in RNA sampled at the six pictured timepoints. The three genes are expected to attain their highest expression during the indicated phases of conversion and gametocytogenesis in the figure. (B) Expression in PB-60, NF54, and F12 of the two markers potentially disrupted in the PB-60 line, *Pf13_0164* and *MAL13P1.163*. For *MAL13P1.163*, the mean of two biological replicates is shown. For *Pf13_0164*, the mean of two biological replicates, or in some cases one biological replicate is shown. Foldchange was calculated using the F12 10 hpi sample as the reference timepoint. Asterisks indicate samples in which there was no amplification of the marker. (C) Expression of three early gametocytogenesis markers in PB-60, NF54, and F12. Each graph displays the mean foldchange of two independent biological replicates relative to the F12 10 hpi value. Asterisks indicate samples with no amplification of the marker.
Figure 6. (Continued)
qRT-PCR timecourses, as the fourth timepoint (58 hpi) includes a significant number of delayed trophozoites and schizonts in addition to rings, and the cells visible in the final two timepoints also appear younger than expected.

*Expression of potentially disrupted genes in high gametocyte producing mutant PB-60.* Results of the quantitative RT-PCR experiments indicated that a peak of *Pf13_0164* expression appears at hour 42 (Figure 6b) in both F12 and NF54. *Pf13_0164* transcript is reduced in PB-60 in relation to NF54 and to F12 (mean 1/8th of F12 expression and 2/5th of NF54 expression; at the 42 hour peak 1/4th of NF54 and 1/5th of F12 expression). There is however a small amount of RNA transcript detectable in PB-60. To exclude nonspecific amplification, amplicons from positive qRT-PCR wells of NF54 and PB-60 were electrophoresed side by side with PCR amplicons from *P. falciparum* genomic DNA on a gel, and all had the correct length. The other potentially disrupted gene in PB-60, *MAL13P1.163*, does not show decreased expression in PB-60 relative to NF54 (Figure 6b), though it had slightly lower expression levels in PB-60 relative to F12 (mean 2/5th of F12 expression level). Thus, the phenotypes observed in PB-60 are likely due to the decreased expression of *Pf13_0164* caused by the disruption of its promoter.

*Expression of gametocyte genes in three strains.* In the timecourses with wild type NF54, results showed that the genes *PFL1085w* (AP2-G), *Pf14_0744*, and *Pf14_0748* are indeed expressed in sequence (Figure 6c). In PB-60, these genes are expressed in sequence but there is a delay in expression of *PFL1085w*, presumed to be due to PB-60’s cell cycle delay. As expected, F12 is unable to induce *Pf14_0744* or *Pf14_0748* and expression of these genes remains very low. F12 does express its mutant copy of *PFL1085w* but no transcriptional peak occurs, likely because the AP2-G positive feedback loop is not functional.
In PB-60, AP2-G expression reaches a higher peak than in NF54, and there is also a second AP2-G expression peak at 120 hpi in the PB-60 population that does not occur in NF54, indicating a subsequent induction in PB-60 independent of the inducing stimulus. This implies that PB-60’s defect is upstream of the increase in AP2-G expression; this idea is also supported by the normal expression of Pf13_0164 in F12, which has an inactivating mutation in AP2-G. Due to the release of HP1 and HDA2 silencing, and due to a positive feedback loop in which it activates its own transcription, the transcription factor AP2-G is expected to be expressed at higher levels in sexually committed schizonts\textsuperscript{18,20,21}. Together these data indicate that the defect in PB-60 leads to de-repression of sexual conversion upstream of AP2-G, and that Pf13_0164 directly or indirectly helps impose the silencing of AP2-G.

2.5. Additional phenotypes of the mutant PB-60.

The mutant PB-60 was previously reported to show a reduction in asexual proliferation to 50% of normal\textsuperscript{34}, which was also observed in my hit retesting experiments. As gametocytes do not replicate, part of this growth impairment can be explained by the loss of 15-20% of cells per cycle to gametocyte development. A cell cycle delay, if present, would also explain a portion of the strain’s growth impairment.

I performed an experiment (Figure 7) to measure length of and progression through the asexual intraerythrocytic replication cycle in PB-60, NF54, and F12. The three strains were tightly synchronized and cultured for 96 hours, equivalent to two cell cycles. At eight hour intervals, slides were made and parasite nuclear content was measured by flow cytometry after
Figure 7. Cell cycle phenotypes of PB-60 and F12 mutants as compared to wild type NF54.

Parasites of the three strains were tightly synchronized to 0-4 hours old by a series of sorbitol synchronizations and purification on a magnetic MACS column. Every eight hours thereafter a sample was stained with the DNA dye SYBR Green and DNA content per cell was measured via flow cytometry. (A) Progression through intraerythrocytic replication cycle. Means of three technical replicates (NF54, PB-60) or one replicate (F12) are shown. The blue line and the green line indicate the proportion of parasites in the culture with DNA content indicating a single genome copy (N=1) or more than one genome copy (N>1), respectively. The black vertical lines indicate the end of each 48 hour period. Red arrows indicate the point in the second cycle at which the parasites with more than one genome copy (trophozoites and schizonts) outnumber the parasites with a single genome copy (rings). (B) Data from the same experiment indicating the timing of progression to trophozoite and schizont stages. Means of three technical replicates (NF54, PB-60) or one replicate (F12) are shown. The magenta line indicates the proportion of parasites which have just completed their first DNA replication (N=2) and the brown line indicates those which have at least four genome copies. Results show that the delay is present in a subset of cells as early as the first DNA replication in each cycle.
Figure 7. (Continued)
staining with the DNA dye SYBR Green. This revealed that while NF54 has a typical \textit{P. falciparum} cycle length of \textasciitilde48 hours, PB-60 has a delay in DNA replication and a lengthening of each cycle by \textasciitilde8-16 hours. The delay, at least in a subset of cells, begins as early as the first DNA replication, suggesting that Pf13_0164 may begin to function by this time; the delay continues to impact later DNA replications (Figure 7b and c). Additionally, F12 may complete its first and subsequent DNA replications slightly earlier on average than does NF54.

\textbf{2.6. Knockdown of Pf13_0164 protein using the destabilization domain tag.}

In order to further investigate the roles and functions of Pf13_0164 and to confirm its involvement in the multiple phenotypes seen in PB-60, I constructed an inducible knockdown of Pf13_0164 protein within wild type NF54 parasites. The destabilization domain (DD) is a mutant of the FKBP protein which can be used to tag the genomic copy of a protein within cells, causing the protein to be degraded by the proteasome. However, a stabilizing small molecule, Shield-1, can be included in the culture medium to prevent this degradation\textsuperscript{40}. DD tagging often allows the experimenter to control the level of protein by varying the concentration of Shield-1; however the intensity of resulting knockdown varies depending on protein characteristics and localization, and the tag itself can disrupt protein function. I constructed two plasmids by cloning \textasciitilde1 kb of the 3’ end of either \textit{Pf13_0164} or \textit{PFF0295c} (disrupted in high gametocyte producer PB-65) coding sequences into the plasmid pJDD145 (kind gift of Dr. Jeffrey Dvorin). The resulting plasmids pJDD-0164 (Figure 8a) and pJDD-0295c are designed to integrate into the genome by single homologous recombination and add a modified 3xHA and a
Figure 8. **Construction of Pf13_0164 knockdown line. (A) Plasmid map.** The plasmid pJDD--0164 is based on pJDD-145 (kind gift of Dr. Jeffrey Dvorin) and is designed for C-terminal tagging of the genomic copy of Pf13_0164 with the destabilization domain (DD) and triple hemagglutinin (3xHA) tags. **(B) Southern blot showing plasmid integration at Pf13_0164 locus.** Genomic DNA from each clone and from wild type parasites (- control) was digested with PvuII and chloroform extracted to remove enzyme. A probe was designed to the DHFR sequence present in the pJDD-0164 plasmid. The positive control in lane 1 is a dilution of the same plasmid. The negative control in lane 2 is genomic DNA from wild type parasites. The subsequent lanes were loaded with DNA from five clones of the transformed NF54/Pf13_0164-DD cells. In the presence of the plasmid the probe binds a fragment of 4,707 bp, while in the integrated locus it binds a fragment of 9,719 bp. Results indicate that Clones 2 through 5 all
Figure 8. (continued)

contain both the plasmid copy and the integrated copy, indicating tagging of the genomic locus.

Too little DNA was loaded for Clone 1.

Author contributions: Plasmids were constructed by I. Goldowitz with the assistance of E. Meyer. Southern blotting was performed by I. Goldowitz and Dr. N. Brancucci.
DD tag to the protein’s C terminus, leading potentially to a protein level conditional knockdown.

I transfected each plasmid into wild type NF54 parasites and obtained drug-resistant parasites; after three cycles on/off the selection drug, followed by cloning by limiting dilution, Southern blot analysis showed that four of five clones of NF54/Pf13_0164-DD have plasmid integration into the genomic locus of Pf13_0164 (Figure 8b). The plasmid for PFF0295c-DD did not integrate into any of five clones after three cycles on/off drug and so was not pursued.

To evaluate knockdowns, protein was extracted from NF54/0164-DD clones after being cultured in the presence of Shield-1 or after 1.5 cycles in the absence of Shield-1, and from wild type parasites, and divided into cytoplasmic and nuclear fractions (See Methods). In both of the NF54/0164-DD clones tested, western blots using anti-HA antibody (Figure 9a) demonstrate that the tagged protein is expressed and is of the expected size. Reduction of protein level indeed occurs when Shield-1 is removed, indicating a successful knockdown. The majority of protein is detected in the cytoplasmic fraction, though a small amount of protein is also detected in the nuclear protein fractions (Figure 9a).

*Phenotypes of Pf13_0164 knockdown parasites.* To measure intraerythrocytic cycle length, parasites of NF54, PB-60, NF54/Pf13_0164-DD Clone 2 (either maintained ON Shield or taken OFF Shield 48h before assay start), and NF54/Pf13_0164-DD Clone 3 (either ON Shield or OFF Shield) were tightly synchronized at Hour 0-4 of the asexual life cycle. Every eight hours for a span of 116 hours (2.5 cycles), DNA content per cell was measured in a sample from the population via flow cytometry, to determine how many times each cell had replicated its
**Figure 9. Phenotypes of NF54/0164-DD clones.** (A) Western blot indicating successful knockdown of Pf13_0164 protein in two NF54/Pf13_0164-DD clones. Cytoplasmic and nuclear protein fractions were extracted from approximately equal numbers of parasites of Pf13_0164-DD clones 2 and 3 after the parasites were cultured in the presence (indicated by +) or absence (indicated by -) of Shield-1, or from wild type 3D7 parasites (- ctrl). The blot was probed using antibodies to 3xHA and to pLDH (Pf3D7_1324900), a constitutive cytoplasmic *P. falciparum* protein, as a loading control. The tagged protein Pf13_0164-3xHA-DD is predicted to be 102.5 kDa, while pLDH is 34.1 kDa. Results indicate that Pf13_0164-3xHA-DD can be found chiefly localized in the cytoplasmic fraction but some protein is also found in the nuclear fraction. Concentrations of the tagged protein are greater when the Pf13_0164-DD parasites are maintained ON Shield than OFF Shield, indicating a successful knockdown, both in the nuclear fractions of Clones 2 and 3 and the cytoplasmic fraction of Clone 3. Too little protein was loaded for the cytoplasmic fraction of Clone 2, as indicated by the lack of LDH staining (red asterisk). (B) Cell cycle phenotypes of Pf13_0164-DD knockdown lines as compared to PB-60 and wild type NF54. Parasites of NF54, PB-60, NF54/Pf13_0164-DD Clone 2 and Clone 3 (either maintained ON Shield or taken OFF Shield 48h before assay start), were tightly synchronized at Hour 0-4 of the asexual life cycle. At Hour 12 and every 8 hours thereafter, a sample of each culture was stained with the DNA dye SYBR Green and DNA content per cell was measured by flow cytometry. The graphs show parasites containing one genome copy (blue) or more than one genome copy (green), as a proportion of the culture. Results indicate a cycle delay very similar to that seen in PB-60 is present in both Pf13_0164-DD clones, both ON and OFF Shield-1. Wild type NF54 completes 3.5 cycles by the end of the experiment, while all the other lines
Figure 9. (Continued)

complete only 3 cycles. Clone 3 of NF54/Pf13_0164-DD produced very similar results to Clone 2 both ON and OFF Shield-1 (data not shown). Vertical black lines indicate the expected start of each 48-hour replication cycle. Red arrows indicate the actual point at which the third replication cycle begins, when the parasites with 1 genome copy (rings) outnumber the parasites with multiple genome copies (trophozoites and schizonts).
Figure 9. (Continued)
genome and how far it had progressed through the cell cycle.

Results (Figure 9b) indicate that a cycle delay very similar to that seen in PB-60 appears in both Pf13_0164-DD clones. This delay appears both in the presence of Shield-1 (when higher protein levels are present by western blot) and the absence of Shield-1 (when lower protein levels are present), and is possibly even stronger in the presence of Shield-1. It is thus likely that addition of the DD and 3xHA tags has rendered the protein non-functional. Additionally, morphological examination reveals that the knockdown strains become quite asynchronous later in the experiment compared to NF54, implying that some cells are more delayed than others or that, in the presence of the knockdown, there is a stochastic aspect to how long a cell spends in each cell cycle phase.

I observed that all of the Pf13_0164-DD clones generated do not produce any gametocytes, both in the presence and the absence of Shield, and regardless of which gametocyte induction procedure is attempted. I generated 8 more clones from an earlier frozen stock of the non-clonal gametocyte-producing bulk population after several rounds of cycling on and off drug selection, and none of the resulting clones produce gametocytes. A complete lack of gametocyte production capability is typically seen with genetic losses such as those within the Chromosome 9 locus that includes gene $GDV1^{41}$ or with mutations in the gene encoding $AP2-G^{18}$. I hypothesized that the Pf13_0164-DD clones have a compensatory mutation causing genetic loss of gametocytogenesis. To test this hypothesis, Clone 2 was sent for whole genome sequencing along with its parent strain to look for unknown or, especially, known
mutations that cause loss of gametocytogenesis. If so, this would be a case of epistasis in which a compensatory mutation partially rescues the severe growth defect or lethality stemming from high or even 100% gametocyte production in a Pf13_0164 deficient strain. The compensatory mutations would thus be strongly selected for, and if Pf13_0164 is essential in asexual cells, it would be impossible to generate an integrated, DD-tagged line in a gametocyte producing strain. A similar example was reported by Cheng et al. (2008)\textsuperscript{42}.

2.7. Possible interactions of Pf13_0164 with known participants in the sexual conversion pathway

In light of the above results I hypothesized that the \textit{P. falciparum} gene \textit{Pf13_0164} codes for an active RING-type E3 ubiquitin ligase which acts as a repressor of gametocyte development. I hypothesize too that Pf13_0164 function is essential for maintenance of asexual proliferation, both to ensure cell cycle progression and to repress differentiation into gametocytes in the majority of cells. The epigenetic regulators HP1 and HDA2 cooperate to impose silencing on AP2-G, and their knockdowns led to phenotypes similar to those seen in PB-60: high gametocyte production, cycle delay or arrest, and slowing or arrest of proliferation\textsuperscript{20,21}. \textit{Pf13_0164} could thus function by ubiquitinating HP1, HDA2, or another unknown protein in the sexual conversion pathway, leading to repression of AP2-G. To investigate possible relationships between Pf13_0164 and HP1, I extracted proteins from synchronized cultures of NF54, PB-60, and NF54/Pf13_0164-DD maintained ON or OFF Shield
Figure 10. Reduced concentrations of HP1 protein in nuclear fractions of Pf13_0164 deficient cells. (A) Western blot of nuclear protein fractions simultaneously labeled with antibody to HP1 (top) and loading control histone H3 (bottom). (B) Western blot of cytoplasmic fractions labeled with antibody to HP1 (top). The same blot was re-probed with antibody to LDH as a loading control (bottom). Samples 1 and 5: NF54. Samples 2 and 6: Pf13_0164-DD ON Shield. Samples 3 and 7: 0164-DD OFF Shield. Samples 4 and 8: PB-60. Note that Samples 6 and 7 were accidentally transposed on the cytoplasmic blot in (B). Samples were extracted at 38-46 hpi (Samples 1-4) and at 46-54 hpi (Samples 5-8). Predicted band sizes are as follows: HP1: 31.02 kDa. Histone H3: 11.14 kDa. Lactate dehydrogenase (LDH): 34.1 kDa. HP1 antibody was a kind gift of Dr. Till Voss.

These preliminary results show that in both the 38-46 hpi and 46-54 hpi time windows, the concentration of HP1 protein is reduced in the Pf13_0164-DD nuclear extracts (Samples 2, 3, 6, 7) compared to NF54 (Samples 1, 5), despite loading of similar or higher amounts of protein in the Pf13_0164-DD extracts. HP1 concentration is even more strongly reduced in the PB-60 extracts (Samples 4, 8). A faint unknown band labeled with HP1 antibody is also visible at approximately 62 kDa in only Samples 6 and 7. In the NF54 cytoplasmic fractions, as expected, no HP1 is present, however a band of ~30 kDa is visible in the 0164-DD OFF Shield lane from 46-54 hpi (Sample 7) and may be visible in PB-60 (Sample 8). No monoubiquitination or clear polyubiquitination mark is visible on HP1 in any of the extracts, however these marks are often very transient and are unlikely to be measured unless they are artificially stabilized.
Figure 10. (continued)
when the parasites were 38-46 hours post invasion (hpi) and when they were 46-54 hpi. Nuclear fractions (Figure 10a) and cytoplasmic fractions (Figure 10b) of these extracts were loaded on western blots and stained with antibodies to HP1\textsuperscript{21} and to histone H3 or pLDH as loading controls. Preliminary results indicate reduced levels of HP1 protein in the nuclear fractions extracted from the Pf13_0164-DD parasites as compared to wild type parasites at both 38-46 hpi and 46-54 hpi timepoints. PB-60 cells show an even stronger reduction of HP1 protein level. In the cytoplasmic fraction, HP1 protein is detected only in the Pf13_0164-DD parasites maintained OFF Shield at 46-54 hpi, and as expected is not detected in the wild type parasites. The reduction in nuclear HP1 is present in all the strains believed to be deficient in functional Pf13_0164 protein, and thus Pf13_0164 activity may be necessary to maintain HP1 presence in the nucleus. A caveat is that the cycle delay could cause the peak of nuclear HP1 protein levels to be missed in the knockdown and mutant strains; therefore the connection between Pf13_0164 and HP1 will need to be confirmed by another method.

2.8. Chapter Discussion.

\textit{Pf13_0164 gene and protein characteristics; E3 ligases in P. falciparum and other eukaryotes}

The Pf13_0164 predicted protein contains three predicted domains, a RING at aa663-721, LisH at aa456-488, and CTLH at aa494-551. RING (also known as RING finger) is the catalytic domain of the largest family of E3 ubiquitin ligase enzymes; it typically binds two zinc ions and interacts with protein substrates and sometimes with nucleic acids. LisH (also known as Lis1) domains are thought to be involved in protein binding and dimerization\textsuperscript{43}, and have
been found in E3 ligases as well as proteins handling microtubule dynamics, nucleokinesis and chromosome segregation. The first half of the Pf13_0164 coding sequence consists of a low complexity region, including strings of asparagine and lysine repeats which are common features of *P. falciparum* proteins; this has likely contributed to the high level of difficulty I experienced in cloning this gene, as the DNA itself appears to be toxic to several *E. coli* strains (see Section 2.9). Yeast two hybrid interactions have been found with two proteins of unknown function and with Pf10_0268 (Pf3D7_1027300), a peroxiredoxin which associates with chromatin throughout the genome, especially on coding regions.

E3 ubiquitin ligases are responsible for the last step in the ubiquitination (or ubiquitylation) pathway. First, an E1 ubiquitin activating enzyme is loaded with ubiquitin, then transfers the small protein to an E2 ubiquitin conjugating enzyme. Next, an E3 ligase binds both the E2 and a substrate protein and catalyzes the transfer of ubiquitin to a lysine residue on the substrate protein. In the larger of the two classes of E3 ligases, a RING domain is responsible for the catalysis. Most genomes contain one or two E1s, a small number of E2, and hundreds of E3 enzymes which bind to and act on different substrate proteins at different times and places. Some E3 ligases function in polyubiquitination (addition of a chain of >3 ubiquitin units), which often signals for destruction of the target protein by the proteasome, and their first discovered role was in cellular “recycling”. Others function in mono- and poly-monoubiquitination (monoubiquitination on multiple sites) which can activate, recruit to DNA sites, or cause a change in localization of the target protein or signal for its destruction. Ubiquitin marks are a post-translational modification that can be applied and take effect very quickly, for example by triggering the rapid destruction of a protein or its change in localization. E3 ligases thus have
special importance in eukaryotic cell cycles and in signal transduction\textsuperscript{46}.

The cell cycle delay of the Pf13_0164 deficient lines is suggestive. E3 ligases play central roles in cell cycles, such as in the anaphase-promoting complex or cyclosome (APC/C) whose main role, at least in animals and yeast, is to trigger progression from metaphase to anaphase when its RING containing subunit polyubiquitinates several proteins used during metaphase, leading to their destruction by the 26S proteasome. Other major participants are the SCF, an E3 ligase complex that helps control the G1/S and G2/M transitions, and the mammalian RING1B that ubiquitinates histone H2 during interphase\textsuperscript{46}.

A genomic study predicted that over 50 E3 ligase proteins may be encoded in the \textit{P. falciparum} genome; along with over 60 other proteins involved in ubiquitination, deubiquitination, and addition and removal of related posttranslational marks\textsuperscript{47}; the predicted E3s include a homolog of the catalytic RING subunit of APC/C, and a homolog of a protein involved in histone ubiquitination. \textit{P. falciparum} E3 ligases appear to be quite diverse and divergent from those of other species, which could make them attractive drug targets\textsuperscript{48}.

\textit{Where does Pf13_0164 act? Potential interaction partners and model of the pathway.} Pf13_0164 is likely part of the mechanism that represses gametocyte conversion and allows asexual replication to proceed in non-sexually committed cells. This could make it essential for continued asexual cycling. The appearance of both a fivefold increase in gametocyte conversion rate and a significant cycle delay in the PB-60 mutant suggests that the protein acts either directly on HP1, on HDA2, or close to those genes in the same pathway, helping to impose silencing on the AP2-G genomic locus and other loci (model, Figure 19). The finding that AP2-G expression is strengthened in PB-60 supports the hypothesis that Pf13_0164’s role in
gametocyte conversion occurs upstream of AP2-G. The normal expression of Pf13_0164 in the AP2-G deficient line F12 could also support this hypothesis.

Likewise, the preliminary data shown in Figure 10 supports the idea that Pf13_0164 regulates HP1, either directly by addition of a ubiquitin mark, or through an intermediate protein. This could potentially support the presence of HP1 in the nucleus, by causing a translocation from cytoplasm to nucleus or through some other mechanism (Figure 19). In some species, HP1 is regulated, either positively or negatively, by RING-containing E3 ligase proteins. In *Schizosaccharomyces pombe*, the HP1 protein Swi6 is ubiquitinated by the APC/C E3 ligase complex, which recruits it to heterochromatin loci, helping to control the cell cycle\textsuperscript{49}. Another group showed that Swi6 is regulated by a second E3 ligase, Msc1, which enables mating type switching by recruiting Swi6 to mating type loci in the genome\textsuperscript{50}. *P. falciparum* HP1 may be a likely candidate for direct regulation by *Pf13_0164* activity (see model, Figure 19).

Another study showed that a *P. falciparum* ApiAP2 transcription factor was marked by ubiquitin\textsuperscript{51}.

*Timing of Pf13_0164 activity and of the events of sexual conversion; possible co-regulation with the cell cycle.* It has been unclear whether *Plasmodium* blood stages go through the classical cell cycle stages and checkpoints that occur in animals\textsuperscript{52}. They likely lack the G2 phase and its checkpoint, but instead go through an initial G1 phase followed by multiple rounds of DNA synthesis (S phase) followed immediately by mitosis (M phase) without cytokinesis. In schizogony, DNA replication can proceed asynchronously, leading to a merozoite number that is not a power of 2, finally followed by one synchronous round of mitosis and
cytokinesis. This appears to be controlled by a balance of global and local regulation at the level of individual nuclei\textsuperscript{52}.

My expression results (Figure 6) and previously reported results\textsuperscript{37} indicate that Pf13_0164 transcript in the asexual cycle peaks at or near the late trophozoite/early schizont stage when DNA replication is very active. During the first replication cycle in the 0164-DD cycle length experiment (Figure 9b), the majority of cells have attained N\textgreater{}1 genome copies \textasciitilde{}4 hours earlier in NF54 than in PB-60, while the majority of cells attain N\textgreater{}4 genome copies \textasciitilde{}8 hours earlier in NF54 than in PB-60. This widening gap suggests that both the first and subsequent DNA replications are progressively delayed. Interestingly, F12 has a similar overall cycle length to NF54, but a greater proportion of parasites undergo their first DNA replication earlier than in NF54, and the progression to schizonts with four or more genome copies occurs earlier in F12 than in NF54 (Figure 7b). A caveat is that F12 is not isogenic with NF54 but is a mutant of the 3D7 reference strain, which was originally cloned from NF54. The cycle length phenotype of 3D7 would need to be carefully evaluated to determine whether F12’s AP2-G mutation might be responsible for this apparent cell cycle phenotype.

The above results point to the suggestion that key steps in the conversion decision might occur at the time of the first DNA replication, or during each DNA replication. The G1 to S transition is delayed in HDA2 knockdown cells\textsuperscript{20} and is the point at which cells arrest in HP1 knockdown cells\textsuperscript{21}. Speculatively the decision to convert could be intertwined with cell cycle progression, similar to cell fate/differentiation decisions that are made at cell cycle checkpoints in other organisms. Pf13_0164 was also found to have a second peak of expression during the
late gametocyte stage\textsuperscript{38}. The protein could potentially be involved with processes that occur in the late gametocyte in preparation for the following cell cycle in which gametes form.

It is thus possible that the central processes of differentiation and cell cycle progression share mechanistic components. Interestingly, several studies in the apicomplexan parasite \textit{Toxoplasma gondii} tell a similar story to the above. This parasite switches between a fast-replicating tachyzoite and a slow-replicating, quiescent bradyzoite form; the latter is the precursor to the tissue cyst which is adapted for both transmission to new hosts and persistence in the same host. Although the \textit{T. gondii} life cycle differs from that of \textit{Plasmodium} because it inhabits mammals as both its definitive and intermediate hosts, there are similarities in the ecological roles and differentiation triggers of the transmission stages of the two parasites. Jerome et al. (1998)\textsuperscript{53} and Radke et al. (2003)\textsuperscript{54} report that a slowdown in tachyzoite replication and a uniform lengthening of the cell cycle in the tachyzoite population occur just before, and are essential for, the initiation of bradyzoite formation in a subset of the population. These delayed tachyzoites are proposed to go through an additional G2 phase (not usually present in apicomplexans), suggesting that bradyzoite differentiation could be dependent on a cell cycle checkpoint that integrates environmental information. Interestingly, A \textit{T. gondii} mutant that was unable to form the slower growing bradyzoite stage became much more virulent in mice than its parent strain\textsuperscript{53}.

\textbf{2.10. In progress and near future experiments}
Because the effect of the Pf13_0164 knockdown on gametocytogenesis is not straightforward, additional confirmation of the gene’s role in this pathway will be very helpful. To further confirm the protein’s role in conversion and its activity as an E3 ligase, several experiments are underway or planned for the near future in the Marti Lab.

First, several strains have been sent for whole genome sequencing: PB-60, NF54/Pf13_0164-DD, and the wild type NF54 used in these experiments. To confirm that the observed PB-60 phenotypes are only due to the known piggyBac insertion in PF13_0164, we will look for other mutations (besides the transposon insertion) in the mutant’s genome, relative to its parent, which may have arisen during creation of the mutagenized library. The whole genome sequencing results will be analyzed to search for mutations that could explain the loss of gametocyte production in the NF54/Pf13_0164-DD knockdown clones, and to search for other mutations in PB-60 that could be alternative explanations for the high gametocyte production phenotype. I consider the latter unlikely, because no genetic mutations are yet known which cause increases in gametocyte production, though there are several that cause loss of gametocyte production.

Second, to determine whether Pf13_0164 codes for an active E3 ubiquitin ligase, I am collaborating with Dr. Ulf Ribacke to express Pf13_0164 protein in E. coli and test the in vitro protein activity in an enzyme assay. Dr. Ulf Ribacke assisted in the design of four peptides for expression which include either the RING domain alone, the region including the three predicted domains, the C terminal half of the protein excluding the low complexity region, or the full protein. Construction of expression plasmids by replacing the insert in pQE30-NFAT5-DBD (Addgene; Dr. Angana Rao) is underway. Any successfully expressed peptide will be
purified and used in an activity assay along with commercial E1 enzyme and substrate and a *P. falciparum* E2 enzyme from Dr. Ulf Ribacke.

Third, to establish the localization of Pf13_0164 and better understand any interaction with HP1, I will perform immunofluorescence staining with antibodies to 3xHA alone or to 3xHA and HP1 on NF54/0164-3xHA-DD cells. By examining these cells microscopically I will identify the protein’s location and whether it co-localizes with HP1.

Fourth (optionally), microarrays may be performed on RNA extracted from NF54, PB-60, and NF54/Pf13_0164-DD parasites ON or OFF Shield-1 at multiple points throughout their cell cycles. As the proteins HP1 and HDA2 also regulate *P. falciparum var* (PfEMP1) genes which are involved in host erythrocyte cytoadherence and immune evasion, it will be especially interesting to determine whether var gene dysregulation similar to that seen in HP1-DD microarrays occurs in those of PB-60 and the Pf13_0164-DD knockdown.

Fifth (optionally) replacement of the wild type copy of the disrupted gene (on a plasmid) within a mutant strain is expected to restore the wild type phenotype if the disrupted gene is indeed responsible for the phenotype, known as genetic complementation. Overexpressing the gene from a plasmid should either restore the parental or reverse the mutant phenotype. I attempted to construct complementation and overexpression plasmids for the gene disrupted in each of the four hit strains, with expression driven by the gene’s native promoter or by the strong promoter for PfHSP86, respectively. Overexpression plasmids were successfully constructed for strains PB-5 (gene *Pf11_0431*) and PB-65 (gene *PFF0295c*) in the *P. falciparum* vector pBIC015 (kind gift of Dr. Bradley Coleman, citation?), but only the plasmid for *PFF0295c* produced live cells after it was transfected into PB-65 cells by Dr. Min Zhang (USF). Despite
numerous attempts using several strategies, a complementation or overexpression plasmid for PB-60 and PB-30 could not be constructed. In the case of PB-60, I was able to clone the coding sequence, an overexpression promoter, and a 3’ utr into a cloning vector (pGEM-T Easy, Promega) but was unable to transfer it to a vector for \textit{P. falciparum} transformation; every step was extremely difficult because the Pf13_0164 DNA was apparently toxic to two \textit{E. coli} strains. Similarly, Ikadai et al. (2013)\textsuperscript{31} state that, despite multiple attempts at cloning and plasmid construction to confirm the 16 hits from their gametocytogenesis mutant screen, they were able to create complementation plasmids for only five of the shortest of these genes; four out of these five successfully complemented the defect and rescued the phenotype.

I plan to test the PB-65 overexpression strain’s gametocyte production phenotype within the format of the original mutant screen. Dr. Nicolas Brancucci (optionally) will attempt to insert the cloned Pf13_0164 sequence into an alternative \textit{P. falciparum} vector for overexpression in the PB-60 line. If Pf13_0164 is indeed responsible for PB-60’s phenotype of ~20% conversion, the resulting overexpression is expected to either restore the wild type conversion rate of approximately 4%, or to reduce it to lower than the wild type.

\textbf{Materials and Methods}

\textit{Parasite culture.} NF54 and piggyBAC mutant parasites were obtained from the laboratory of Dr. John Adams (University of South Florida). Parasites were cultured in Albumax culture medium as described (Buchholz et al. 2011). Synchronization to obtain ring stages was carried out by incubating parasites for 10 minutes at 37°C in 5% sorbitol/water, followed by two washes in MCM. Synchronization to obtain schizont stages was carried out using MACS LS
(Miltenyi Biotec) magnetic columns according to the manufacturer’s instructions. For the GIA, 220ul of the appropriate culture mix was added per well in 96 well plates; for the cell cycle length experiments 110ul of culture were added per well. Cells for the RNA collection timecourses were cultured in TC-treated T75 flasks. Cells for the cycle length experiments were cultured in 10 ml dishes and were kept in a shaking incubator to reduce double invasion of RBCs. For all experiments with Pf13_0164-DD cells, parasites were maintained with 4 nM WR 99210 (Jacobus Pharmaceuticals), and ON Shield parasites were cultured in 625 nM of Shield-1. OFF Shield parasites were washed carefully 4x when removed from Shield.

*Gametocyte immunofluorescence labeling, parasite DNA dye labeling and flow cytometry.* To obtain the parasitemia readings in the initial screen, a 30 ul sample of culture from each well was stained with the DNA dye SYBR Green diluted 1:2000 in RPMI by incubating at room temperature for 20 minutes, followed by two washes. To obtain the gametocytemia readings in the initial screen, 150 ul of culture from each well was fixed using a 4% paraformaldehyde (Electron Microscopy Sciences), 0.0075% glutaraldehyde solution in PBS, permeabilized using 0.1% Triton X-100 (Calbiochem 648466 Molecular Biology Grade) in PBS, then blocked in 3% BSA (CalBioChem 2930 OmniPur BSA Fraction V) in PBS. Gametocytes were labeled with the gametocyte specific Pfg27 antibody (kind gift of Dr. Kim Williamson) and secondary Alexa Fluor 488 antibody, essentially as described in Tonkin et al. (2004)\(^5\). Flow cytometry was performed on both parasitemia and gametocytemia samples by reading 50,000 or 200,000 events, respectively, in a forward scatter/side scatter RBC gate on an Accuri C6 flow cytometer in the Adams Laboratory at USF. Data was analyzed using Accuri C6 software.
For the retesting experiments, to obtain the parasitemia readings, 30 ul of culture from each well was stained with 1:4000 Hoechst/PBS by incubating for 30 minutes at 37°C, followed by two washes in PBS. Flow cytometry was performed using a Beckman Coulter Cell Lab Quanta SC, with an ultraviolet light source and a 465/30 nm band pass filter. 50,000 events were detected on a forward scatter/side scatter trigger in the Marti Laboratory. Data was analyzed using FlowJo software version 8.8.6. Due to flow cytometry problems, gametocytemia measurements were obtained by counting Stage II gametocytes per ~20,000 RBC.

In both sets of experiments, gates for the parasite population were drawn using the Hoechst (UV) or FITC (green) channel and the forward scatter channel; gates for the gametocyte population were drawn using the FITC channel. In both cases, gates were selected to ensure that the appropriate negative control (uninfected RBC for parasitemia and gametocyte-null F12 parasites for gametocytemia, both incubated in the plate for the duration of the experiment) has very low background. Uninfected RBC background for each plate was then subtracted from both parasitemia and gametocytemia results.

In cell cycle length experiments, 10ul of parasite culture was removed at each time point, stained as above in SYBR Green/PBS and washed 2x. To determine DNA content per cell, flow cytometry was first tested on mixtures of stained 3D7 cells of known ages to obtain gates performed on a Miltenyi Biotec MACS Quant VYB flow cytometer FITC channel. Parasite populations with 1, 2, 3, and 4 or more genome copies could be clearly distinguished; these gates were later used to quantify DNA content in the experimental cells along with uRBC controls. The exception is for the first and third timepoints in the first cycle length experiment (Figure 7), for which Giemsa slide staging of parasites was performed to estimate DNA content;
since cells were rings at the time and were known to be tightly synchronized at experiment start this does not affect the interpretation.

*Quantitative RT-PCR.* Primers were designed using the PrimerQuest tool ([www.idtdna.com](http://www.idtdna.com)) and purchased from IDT. BLAST searches were performed against the *P. falciparum* genome to ensure each expected amplicon is specific to the gene of interest. 10-fold dilution standard curves covering 9 logs were performed with *P. falciparum* genomic DNA of known concentration on the Viia7 RT-PCR system. Primer efficiencies were calculated based on the slope of the curve over the linear range, and the best pair for each gene was selected. Limits of detection were determined by estimating the number of genome copies present at the lowest linear point on the curve (Table 2). All $R^2$ values for standard curves were 0.99 or greater. When amplicons were analyzed on agarose electrophoresis gels, each primer pair produced a single band of the expected length from both genomic DNA and complementary DNA, and amplicons greater than 110 bp in length were sequenced to confirm their identity.

Timecourse culture samples were preserved in Tri Reagent (Molecular Research Center) and stored at -80°C. RNA was extracted by phenol-chloroform extraction and purified using Qiagen’s RNEasy Plus Kit according to the manufacturer’s instructions. RNA quality was tested using NanoDrop spectrophotometry. Complementary DNA was synthesized using the SuperScript III first-strand synthesis system and random hexamer primers followed by RNAse H digestion of remaining RNA (Life Technologies). Quantitative RT-PCR was performed on the Viia 7 (Applied Biosystems) qRT-PCR system with each sample assayed in triplicate. No Reverse Transcriptase and No Template negative controls were included in each plate. Baselines for
threshold cycle (CT) analysis were set using ViiA 7 software (Applied Biosystems), and all thresholds were set at 0.175, which was adequate for all samples with positive amplification.

Samples (triplicates or when appropriate single wells) were excluded from analysis and flagged for repetition if they had any of the following: amplification in No Reverse Transcriptase and No Template negative controls fewer than six CTs away from the sample (or five CTs for those with positive amplification above CT 38); triplicate standard deviations higher than 0.4; or significant secondary melting points. Relative quantification was performed for each sample using UCE as the endogenous reference gene with correction for the efficiency of each primer pair\(^{39}\) followed by calculation of fold change values.

**Cloning.** To create the plasmid pJDD-0164, ~1 kb of the 3’ end of Pf13_0164 coding sequence was amplified from NF54 genomic DNA and cloned into pJDD-145. Sequencing reactions (Genewiz Inc.) showed the insert was in the correct location and the DD tag sequence was correct.

**Southern blots.** DNA samples were digested using PvuII enzyme, then chloroform extracted to remove enzyme. Approximately 1.2 ug of DNA per sample were loaded on a 0.8% agarose/TAE gel and electrophoreed at 40V for five hours. DNA was transferred to a nylon membrane (Amersham Hybond-N, GE Healthcare) and labeled with P32 radiolabeled probes prepared from DHFR PCR products.

**Protein extraction and western blots.** After saponin lysis of erythrocytes and recovery of parasites, parasites were lysed for cytoplasmic protein extraction, and nuclei were lysed using a 1M KCl based nuclear extraction buffer. Protein samples were diluted with NuPAGE LDS Sample Buffer and heated at 70°C for 10 min, then 0.05M dithiothreitol was added. Samples were
loaded on NuPAGE 4-12% Bis-Tris protein gels (Life Technologies) and electrophoresed in MOPS buffer for 50 min at 200V. Proteins were transferred to a PVDM membrane using a wet blot protocol. The membrane was labeled using antibodies to PfHP1 (kind gift of Dr. Till Voss) and to either parasite histone H3 (nuclear fraction) or to parasite lactate dehydrogenase (cytoplasmic fraction) as loading controls, then was secondarily labeled with Licor 800CW and 680RD antibodies and photographed using a Licor Odyssey imager.
Chapter 3: Parasite density and parasite-derived factors impact the gametocyte conversion pathway.
3.1. Chapter Introduction.

Sexual conversion is a key decision point in the malaria life cycle. In order to optimally transmit from their human host to their mosquito vector, malaria parasites might be expected to alter their sexual conversion rates, and thus their gametocyte production, in response to conditions that impact asexual growth and gametocyte viability. These conditions could include parasite density, nutrient availability, the presence of drugs, host clinical status, and host immune responses, among others. Indeed, researchers have long reported that environmental conditions parasites encounter have a major influence on control of sexual conversion. As early as 1979, soon after the development of in vitro *P. falciparum* culture, Carter and Miller reported that parasite strains vary their gametocyte production according to the culture environment and those kept in continuous culture without dilution eventually produce gametocytes in bursts. In vivo, gametocyte production or carriage is reported to change through the course of an infection and may be enhanced in relation to drug treatments, high asexual parasitemia, fever, and patient clinical status.

A wide array of environmental conditions and treatments, mostly categorized as “stress”, have been reported to stimulate gametocytogenesis in experiments. These include high asexual parasitemia, sublethal concentrations of several antimalarial drugs, other toxic molecules such as ammonium compounds and the veterinary anti/protozoal agent Diminazen, and endoplasmic reticulum stress induced by DTT treatment (e.g. Ono et al., Chaubey et al.). Additionally, several molecules that may target or mimic classic components of eukaryotic signaling pathways are reported as gametocyte-inducing stimuli: cholera toxin, which activates cAMP-dependent pathway and protein kinase A in animal cells; cyclic AMP and 8-bromo-
cAMP; and phorbol diester, a protein kinase C activator and diaclylglycerol mimic; many conserved components of signaling pathways have in fact been identified in *P. falciparum*. The latter could potentially provide mechanisms for the parasite to sense and respond to external conditions in the host or internal conditions of parasite cell health.

One of the most commonly reported gametocyte-inducing stimuli is high asexual parasitemia. Additionally several reports have indicated that parasite-conditioned medium from high parasitemia cultures stimulates gametocyte production *in vitro*. Considering that parasite-conditioned medium and high parasitemia are commonly used as gametocyte-inducing stimuli for in vitro production *e.g.* I first hypothesized that parasites are able to a) detect each other’s presence and density through parasite-induced changes in the medium, and b) use this information to alter their gametocyte production in response to conditions relevant to survival and transmission. Detection could occur through secretion of a parasite-derived factor and/or through depletion of nutrients from the medium.

Evidence for environmental control of sexual development in diverse prokaryotic and eukaryotic species suggests that parasite density or adverse environmental conditions could similarly play a role in *P. falciparum*. In species that can choose flexibly between pathways for reproduction and development, environment is often a controlling factor. Adverse environments often trigger sexual maturation or mating behavior. Similarly, many species across kingdoms form cysts, spores, and other dormant forms when under stress. These forms can either transmit to a new environment or persist until conditions improve. Conversely, favorable environments often lead to asexual reproduction, which allows a clonal population to more quickly exploit resources. Quorum sensing refers to systems in which microbes regulate
group behaviors in relation to population density and/or size; the density information is commonly carried by small molecules or peptides released by the individual cells. Quorum sensing is best understood in bacteria, in which N-acyl homoserine lactones and small peptides are the most common mediators of density detection, but similar phenomena have been reported in eukaryotic groups, including trypanosomes, amoebas, and even some animals.

*C. elegans* is a nematode whose ecological strategy relies on quickly exploiting an available food resource by producing large population expansions, then surviving for long periods in the “dauer” form, a metabolically quiescent, arrested larval stage which is believed to be responsible for dispersal to new locations. Dauer formation was found to be induced by low food availability and especially by conditioned medium in which worm populations had already been cultured. The latter was shown to induce dauer formation when the local population reaches a certain size in a quorum sensing-like system. The response is mediated by Dauer-inducing pheromone, an ascaroside small molecule which is continually released by the nematodes and whose concentration is sensed as a proxy of local population size and density.

Similarly, the social amoeba *Dictyostelium discoideum* uses density sensing to trigger aggregation of thousands of individual cells to form a fruiting body when the local population reaches a threshold density; the group behaviors are regulated by secreted cAMP and Differentiation Inducing Factor, a hexaphenone (reviewed). In infections of the kinetoplastid parasite *Trypanosoma brucei*, fast-growing “slender” forms in mammalian bloodstream transition to quiescent, cell cycle arrested “stumpy” forms when the population density passes a threshold. The formation of these “stumpy” forms, which are thought to be responsible for transmission to the tsetse fly vector, is regulated by an unknown parasite-derived factor called
Stumpy Inducing Factor (SIF). The response to SIF is thought to be mediated by cAMP-dependent signaling (reviewed\(^75\)). Like \textit{P. falciparum} sexual conversion, this system differs from classic quorum sensing because only a subset of cells become stumpy forms at one time instead of the entire population. As both are virulent mammalian blood parasites vectored by biting insects, the ecological role of the transmission stage is similar in these two parasites and their regulation by population density and environmental conditions could follow a similar logic.

The \textit{P. falciparum} gametocyte is both the sexual form and the transmission form, and while not truly dormant, it is more durable and longer-lived than asexual blood stages. Mature gametocytes are also more resistant to certain drugs and stressors that easily kill asexual parasites. It could be important for the parasite to monitor environmental conditions and the state of the parasite population to ensure the best allocation of resources to sexual development and transmission vs. population growth, and to ensure the survival of the population through hard times. Adjustment of developmental decisions at the optimal times could occur in part through parasite density information carried between cells by small molecules, peptides or other factors. Regulation of this process could be similar to quorum sensing regulation of quiescent and transmission stages in other eukaryotic groups.

To investigate the possibility that a factor released into conditioned medium carries density information between parasites, I used the gametocyte induction assay (Figure 3 in Chapter 2) to evaluate the effect of different conditioned media on sexual conversion and parasite growth. I next investigated the effect of malaria microvesicles which can be purified from conditioned medium and are a candidate for the carrier of its effects. I also aimed to
investigate whether parasites alter their sexual conversion rate in relation to conditions that constrain asexual growth.

**Results**

**3.2: A gametocyte-inducing factor is released by parasites into conditioned medium, chiefly during the final hours of the asexual life cycle.**

I performed experiments to treat 3D7/164-GFP parasites with parasite-conditioned medium during hours 20-48 of their life cycle in the GIA (Figure 3 in Chapter 2). To generate conditioned medium, malaria culture medium (MCM) containing human serum was incubated with cultured parasites for 48 hours in the initial experiment, then was applied at 50% strength to the second set of cells as they proceeded from hours 20-24 to hours 48-52 post invasion in the GIA. Control cells were treated with 50% sham-conditioned medium which had been incubated on uninfected RBCs under identical conditions. Total parasite and gametocyte production were measured via flow cytometry after the subsequent reinvasion (Figure 11a). The results confirmed that addition of parasite-conditioned medium increases conversion rate by 1.7fold on average, under the described conditions.

To determine the timing of release of the conversion-inducing factor, I then tested the activity of conditioned medium (CM) generated by incubating on cells during different segments of the asexual lifecycle (Figure 11b, left). Results indicate that the inducing component of conditioned medium is released maximally during the second half of the 48-hour
Figure 11. Conditioned medium from parasites of specific ages stimulates gametocyte conversion. (A) Conditioned vs. control medium. Conversion rates were measured in cultures exposed to 50% fresh medium (blue points and bars) and 50% conditioned medium (green points and bars). Conditioned medium (CM) was prepared by incubation for 48 hours on synchronized parasite cultures at 4% hematocrit and 1 to 3% parasitemia (“0-48 hour CM low”), or 4 to 6% parasitemia (“0-48 hour CM high”). Control medium was sham-conditioned by incubation on uninfected red blood cells under identical conditions. Three (for 0-48 hour CM high) or four (for 0-48 hour CM low) independent biological replicates of the GIA (gametocyte induction assay, see Figure 3 in Chapter 2) were performed on 3D7/164-GFP parasites, with between four and six technical replicates each. Graphs show mean and sem of the fold-change in conversion rates from control, with the biological replicates shown separately (left) or combined (right). (B) Activity of conditioned media from different hours of the asexual life cycle. Left: Conditioned media (green bars) were prepared by incubation on cells for 20 or 24 hour spans on parasites of different ages, all of which were at 4% hematocrit and between 2.3% and 4.5% parasitemia, or by incubation on MACS-purified late trophozoites/schizonts for a span of six hours (egress, purple bar). 50% conditioned medium or 100% control medium (incubated on uRBC, blue bar) was applied to treatment 3D7/164-GFP parasites in the GIA. Mean and sem of two or three biological replicates are shown, each performed in at least technical triplicate. Conditioned medium from 0-24 hour old parasites shows little or no activity. All three of the conditioned media with an inducing effect include cells during the second half of their life cycle and at least some parasite egress. Right: More specifically, while CM prepared on cells from 0-30 hours old has no activity, CM from 30-44 hour old parasites has strong inducing activity as
Figure 11. (Continued)

measured in the GIA. Only a small amount of parasite egress occurred during preparation of the 30-44 hour CM. Both sets of CM-producing parasites were at 4% hematocrit and 7% parasitemia. Gametocytemia was determined by counting gametocytes per 20,000 RBC on slides. Mean and sem of three biological replicates are shown. (C) Activity of conditioned media including or not including parasite egress. Conditioned media were prepared by incubation on cultures of CDPK5-DDTM parasites which were either treated with Shield-1 throughout or were removed from Shield-1 one cycle before experiment start. Only the CM from 25-54 hour parasites maintained ON Shield (rightmost bar) included a significant amount of parasite egress, while the OFF Shield preparation included only a small amount of parasite egress by examination of slides. The CM from 25-54 hour ON Shield parasites does not show a greater inducing activity than the CM from OFF Shield parasites, suggesting that egress is not responsible for the inducing effect seen in (B). The graph shows mean and sem of three (25-54 hour CM) or one (0-24 hour CM) independent biological replicates, each of which was performed in at least technical triplicate on 3D7/164-GFP parasites. (D) Presence of erythrocyte lysate is unlikely to explain the inducing effect. Uninfected (uRBC) or infected (iRBC) erythrocytes were lysed by freeze-thawing and were resuspended in fresh medium at three concentrations; these were then used to treat 3D7/744-GFP parasites in the GIA. The uRBC Low and iRBC Low lysates were resuspended to a concentration equivalent to the volume of erythrocytes lysed when a 7% parasitemia, 4% hematocrit culture egresses (0.28% hematocrit). The mean of two (control and uRBC lysates) or one (iRBC lysates) biological replicate is shown.
Figure 11. (Continued)

**Note:** The data shown in figure 11b (right) has been published in Mantel et al. 2013 *Cell Host & Microbe* where it is shown as Figure 7D (left). This publication is included as an Appendix. I. Goldowitz conceived and performed the experiment under the supervision of M. Marti.
Figure 11. (Continued)
asexual cycle, and especially during the last 14 hours. During these hours cells are in the late trophozoite and schizont stages and are replicating their DNA, or alternatively are undergoing egress, when mature parasites burst from their host RBC. The CM from this time span induced a mean 5.4-fold increase in conversion.

To distinguish release during egress from release during the late trophozoite and schizont stages, I performed experiments using the egress-deficient line CDPK5-DD\textsuperscript{76}. PfCDPK5 is a protein kinase which \textit{P. falciparum} schizonts require to release themselves from their host red blood cell. In CDPK5-DD conditional knockdown parasites cultured without the DD ligand Shield-1, the fully mature merozoites are trapped in the RBC membrane and cannot egress\textsuperscript{76}. I produced CM on synchronized CDPK5-DD cells from hours 0-24 and hour 24-54 of the life cycle in the presence or absence of Shield-1 and tested their effects on 3D7/0164-GFP cells. Results indicated that there was little or no difference between the late stage parasite conditioned media that included egress and those that did not (Figure 11c); therefore parasite egress is unlikely to be responsible for the effect of CM.

It has been hypothesized that erythrocyte lysate could induce gametocyte production\textsuperscript{78}. To further test the possibility that erythrocyte contents released during parasite egress are responsible for the CM effect, I lysed cells using three freeze-thaw cycles and added uninfected or infected erythrocyte lysate to cells in the GIA. The lowest lysate concentration tested was resuspended to 0.28% hematocrit of lysed cells, which would occur from egress of 7% parasitemia cells cultured at 4% hematocrit. While the higher concentrations may have an inducing effect, the more physiologically relevant lowest concentration does not (Figure 11d).
This combined with the CDPK5-DD result indicates that erythrocyte lysate is not likely responsible for the CM effect.

To further define the identity of a putative gametocyte-inducing factor in CM, I fractionated conditioned medium generated on cells during the entire 48-hour asexual lifecycle. Results (Figure 12a) demonstrated that the factor can be concentrated by centrifugation and was found in the pellet after a 15 minute 21,000g spin in a microcentrifuge. Preliminary data also suggests that the factor may be concentrated by filtration and does not pass through a 10 kDa molecular weight cutoff filter. Together, these results suggest that the gametocyte-inducing component in the conditioned medium is part of an insoluble complex or vesicle. Treating cells with increasing concentrations of the pellet resuspended in 100% fresh MCM induced higher conversion rates (Figure 12b, dark grey bars), indicating that the effect of conditioned medium cannot be explained by nutrient limitation, but at least part of the effect must be due to factors released by parasites or iRBCs.

**3.3. Malaria microvesicles purified from conditioned medium strongly stimulate gametocyte conversion and are taken up intact by other parasites.**

Building on evidence for parasite- or iRBC-induced factors in CM that increase sexual conversion, I next collaborated with Dr. Pierre-Yves Mantel (formerly Marti Lab, currently of University of Fribourg) to test the effect of infected RBC-derived microvesicles purified from conditioned medium on sexual conversion rates. Microvesicles (MVs), also known as microparticles, are extracellular vesicles of 100-500 nm that are released by cell membrane blebbing from a wide variety of cell types. MVs have been well studied in mammalian systems:
they are thought to function in cell-cell communication and immune modulation and are particularly important in cancer progression\textsuperscript{79}. Dr. Mantel and our collaborators\textsuperscript{77} showed that malaria microvesicles (iMVs) are released chiefly during the parasite late trophozoite and schizont stages, especially in the last six hours of asexual development; experiments with CDPK5-DD parasites and the egress inhibitor E-64 showed that release occurred before egress from the erythrocyte. These vesicles can be concentrated from conditioned medium by a procedure involving centrifugation at increasing speeds (up to 10,000g), filtration of the supernatant (100 kDa molecular weight cutoff filter), pelleting of the concentrate (100,000g), then flotation of the pellet on a 60% sucrose cushion. We identified these microvesicles as a candidate for the parasite-derived active component of conditioned medium, and hypothesized that treating parasite cultures with iMVs would lead to enhanced gametocyte conversion.

To test this hypothesis, I directly tested the effect of malaria MVs on sexual conversion (Figure 12b, c). Results show that MVs purified from parasite-conditioned medium do stimulate enhanced gametocyte production in treated parasites when they are applied from Hour 0-Hour 48 of the intraerythrocytic life cycle. This effect is dose-dependent across a wide concentration range, and treated cells produced a statistically significant increase (P<0.05) of up to 15fold higher gametocytemia than control cultures. The level of gametocyte induction induced by 16.5 mg/ml iMVs is similar to that produced by 50% conditioned medium in the same plate (Figure 12b). While similar-sized vesicles are released at low levels from healthy red blood cells, they are less abundant than and differ in content and size from those from \textit{P. falciparum}-infected RBCs\textsuperscript{77}. MVs generated from uninfected RBCs (uMVs) using an ionophore treatment produced a
Figure 12. (A) Effectiveness of CM after centrifugation or molecular weight cutoff filtration.

After removing cells, conditioned medium produced by incubation on parasites at 2% parasitemia and 4% hematocrit for the entire 48 hour asexual cycle was centrifuged for 15 minutes at 21,000g to prepare pellet and supernatant samples. Additional conditioned medium was passed through a 10 kDa molecular weight cutoff filter (Centrisart, Sartorius) to prepare MWCO concentrate and MWCO filtered samples. The samples were each used to treat 3D7-0164/GFP cells in the GIA. The mean and sem fold change from uninduced cells of three biological replicates are shown. Because of the low induction of the whole CM sample compared to both the filtered and concentrated MWCO samples, this is preliminary data and would need to be confirmed with fractions of CM from 30-44 hour old cells. (B) Parasite-derived iMVS (black bars) purified from conditioned medium potently and titrably induce gametocyte conversion. Microvesicles were purified as described in Mantel et al. 2013 CHM from conditioned medium which had been incubated on 0-48 hour parasite cultures (iMV), or were generated from uninfected RBCs by application of an ionophore (uMVs). Protein content of the MVs was measured by the bicinchoninic acid method to determine concentrations to add to each well. The MVs were resuspended in fresh medium and applied to cells in the GIA, with the modification that experimental cells were treated from 0-48 hours instead of 20-48 hours, and that gametocytes were quantified microscopically by counting at least 20,000 RBCs per well due to flow cytometry problems. Results show that the iMV strongly stimulate gametocyte production, leading to % gametocytemia up to 15fold that of the control. Also included as treatments in each plate were 50% CM and pelleted CM resuspended in fresh medium at two concentrations; the CM sample produces a comparable effect to iMV at 16.5 ug/ml. The graph
Figure 12. (Continued)

displays mean and sem of three independent biological replicates, each of which includes four technical replicates. Gametocytes produced in the iMV-treated wells are morphologically normal (right). (C) Nonlinear curve fit of gametocytemia vs log iMV concentration (left) in the same experiment shown in (B), performed using GraphPad Prism’s log(agonist) vs response, 4 parameters function. A tentative nonlinear curve fit of parasitemia vs. log iMV concentration was performed to show the presumed trend (right), using GraphPad Prism’s log(inhibitor) vs response, three parameters function, but more data points are needed to confirm this. The inducing effect of iMVs is apparent at concentrations that do not affect asexual parasite proliferation. However at concentrations of 165 ug/ml and higher parasite growth is impaired.

Note: The data shown here in Figure 14B and 14C (left) has been previously published in Mantel et al. 2013 Cell Host & Microbe, as Figure 7D.

Author contributions: I. Goldowitz and M. Marti conceived the experiment, I. Goldowitz performed and analyzed the experiment and took the photographs shown. Pierre-Yves Mantel prepared the microvesicles used in the experiment from conditioned medium and tested their protein concentration. I. Goldowitz prepared the other conditioned medium and CM pellet samples.
Figure 12. (Continued)
slight increase in gametocyte production but this did not show a dose-response relationship and was not statistically significant (P<0.05). Dr. Mantel and our other collaborators also performed a systematic characterization of both iMVs and uMVs, and found that the iMVs contain parasite-derived and erythrocyte-derived proteins; their other contents are unknown. Interestingly, parasite growth was not significantly inhibited by any microvesicle concentration except the highest one (330 ug/ml protein), which caused a dramatic decrease in proliferation/survival while also causing the strongest increase in gametocyte production (Figure 12b, c).

To investigate how iMVs could induce these effects, Dr. Mantel labeled iMVs with PKH67, a fluorescent membrane dye, incubated them with infected or uninfected RBCs, and visualized them by fluorescence microscopy and flow cytometry. Results (reported in Mantel et al. 2013\textsuperscript{77}, see Appendix Figure 7) showed that iMVs appear to be internalized by infected RBCs and by the parasites themselves. In immune electron microscopy experiments, after incubation with iMVs, parasite cells are observed to contain labeled double membrane-bound vesicles which congregate near the nucleus. The vesicles are taken up more efficiently by infected RBC, while they are mainly found bound to the surfaces of uninfected RBC, indicating that they are likely internalized by parasite-specific mechanisms. Though normal RBCs are unlikely to be capable of endocytosis or other uptake mechanisms, malaria parasites make extensive modifications to their host RBCs as they develop\textsuperscript{80}, including the export of hundreds of proteins and the development of a parasite-derived membrane system in the RBC cytoplasm. These systems allow the parasite to take up nutrients from the bloodstream and could potentially be used for iMV uptake.
3.4. Experiments investigating density detection in parasites.

*NanoSight analysis of microvesicle content directly in conditioned medium.* NanoSight (nanoparticle tracking analysis) can produce semi-quantitative concentration and size distribution measurements of natural and synthetic nano-sized particles. Initially, I used a NanoSight instrument to detect microvesicle-sized particles in fresh, parasite-conditioned and RBC-incubated sham-conditioned medium without purification. A large background of microvesicle-sized particles between 0-230 nm, with mode of ~70 nm, was observed in both fresh and conditioned medium samples; comparisons with serum free medium confirm, as expected, that this is due to the inclusion of human serum in the malaria culture medium (MCM). However, additional particles from ~120-250 nm existed in the CM samples that were not present in the fresh medium or uRBC sham-conditioned medium. To reduce the number of microvesicle-sized particles in the MCM, I partially purified pooled human serum using ultracentrifugation and filtration. In experiments using the partially purified serum to prepare fresh and conditioned media (Figure 13a), there is still a large background of particles below 120nm and a small amount of background between 120 and ~230 nm, but it is clear that the parasite-conditioned medium additionally has many additional particles, and I was able to distinguish whole conditioned medium derived from high-, medium-and low parasitemia cultures from each other. These preliminary results suggest that parasites could use microvesicle concentration as a proxy for parasite density. The putative parasite-derived iMVs in the whole conditioned medium range from 120 to 250 nm with a peak at 170 nm (Figure 13a, middle and right), although it is possible that additional peak of particles smaller than 120 nm
Figure 13. (A) Detection of microvesicle-sized particles directly in 3D7 conditioned medium.

The size distribution and approximate concentration of particles <500nm were measured in conditioned medium from PB-60 (NF54) cells, in sham-conditioned medium incubated on uninfected RBC, and in fresh medium, using Nanoparticle Tracking Analysis (NTA) on a NanoSight instrument (NanoSight Ltd). (Left) A large background of particles with sizes centered on 70nm is present in the human serum-based culture medium, but additional particles from 120-250nm are present in the parasite-conditioned medium. (Middle) A detail of the same experiment is shown. (Right) Particles in the 7% parasitemia CM sample after subtracting the fresh medium baseline. Putatively parasite-derived particles between 120-260 nm with a peak at ~170 nm are visible in the 7% parasitemia sample that are not present in the control samples. Each sample was diluted 1:20 in PBS and read on the NanoSight for one minute; detected particles were binned in increments of 1 nm. NanoSight runs in which the reading was visibly distorted during the run because one large particle or one particle stuck to the instrument surface obscured many other particles were excluded from analysis. (B) Conversion rate and percent parasitemia attained during the GIA are negatively correlated among a mutagenized library of isogenic strains. Data from the initial piggyBAC library screen in Chapter 2 (Figure 4) are re-analyzed here. All strains were diluted to an estimated 1% parasitemia in plates, but the starting parasitemia was not measured after plating and is expected to vary slightly due to pipetting error. This smaller variation in the plating parasitemia could have contributed to the conversion rate variability through its effect on density-dependent parasite behavior.
Figure 13. (Continued)

Note: The piggyBAC mutant strain PB-60 was used for (A) because issues with toxic serum had killed my other cultures at the time but PB-60 cells had not yet been exposed. I expect that other strains would provide similar results.

Author contributions: The analysis shown in (C) is a re-analysis of the experiment shown in Figure 4 (B) through (D). See the author contributions note for Figure 4.
Figure 13. (Continued)
could be obscured by the background of similar sized particles in serum-containing medium. This range is close to the size range of 150-250 identified for vesicles purified from parasite-conditioned medium but not uRBC-conditioned medium (Mantel et al. 2013).

In preliminary tests, it was possible to collect MVs from parasites for 2.5 or 5 hours in plain RPMI medium without added serum, while longer incubations in plain RPMI visibly damaged the cells. This observation could allow for future experiments in the Marti Lab in which MVs are collected with no background from the medium.

**Re-analysis of a mutagenized library screen: conditions that restrain asexual growth may enhance gametocyte conversion.** To understand how parasites regulate conversion in relation to parasite density and other stressors that threaten asexual viability and proliferation, I re-analyzed the screen data reported in Figure 4 of Chapter 2. Briefly, 79 clones from a transposon mutagenized library constructed in the strain NF54 were assayed for gametocyte production and total parasite production in a version of the GIA. In these experiments, schizonts were purified by MACS magnetic purification and allowed to reinvade for 4 hours, then remaining schizonts were killed by sorbitol treatment, leaving a synchronous population of 0-4 hour old rings. These cells were accurately counted and diluted to 1% parasitemia and 2% hematocrit at the time of plating (20-24 hours into the conversion cycle of the GIA), by which time they would not have been exposed to a significant amount of iMVs or active CM from the same cycle. 50% fresh and 50% conditioned medium separately grown on the same strain at 4% hematocrit was applied at the time of plating. The CM producing cultures were generally between 1 and 3% parasitemia but this was not tightly controlled. Thus, this screening data represents a set of clonal lines which are isogenic except for a single piggyBAC transposon insertion and were
treated in a standardized assay, but with differing intrinsic growth rates and exposed to differing strengths of CM. I re-analyzed the data to look for relationships between asexual proliferation and gametocyte production during the same reinvasion round (Figure 13b).

If a baseline conversion rate for the strain is the predominant influence on gametocytes produced in the screen, one might expect to see a close relationship between % parasitemia and % gametocytemia produced in the same round. In that case isogenic clones with normal conversion machinery would be expected to have close to the same conversion rates in a standardized assay. Or, if stochastic gametocyte production driven by AP2-G promoter activation accounts for most of the variation, there would be no strong relationship between % parasitemia and % gametocytemia. In both of these cases no correlation between % parasitemia and % conversion would be expected in this library, except in the case of outliers like PB-60 and PB-65 which probably have disruptions of specific regulators of conversion.

Instead, the piggyBAC library reanalysis shows a correlation of -0.49 between % conversion and % parasitemia (Pearson r; p value <.0001). Meanwhile there is only a non-significant weak positive correlation of 0.15 between % gametocytemia and % parasitemia, yet the range of gametocytemia is wide (excluding outliers). This supports an alternative explanation: conditions that restrain asexual growth promote differentiation to gametocytes, while conditions that are favorable for asexual growth suppress differentiation.

The relevant conditions likely include both internal and external conditions of strain health and perceived population density. A strain exposed to CM from a higher density culture could attempt to constrain its growth under perceived adverse conditions, or its asexual
proliferation could be impaired. A “sick” mutant could be more sensitive to stress and this could impact its conversion pathway through stress-sensing pathways or checks, as well as impairing its proliferation. The latter could be a major factor in this experiment as Balu and colleagues (2010\textsuperscript{34}) found that up to 70\% of piggyBAC strains had altered proliferation rates (50\% slower and 20\% faster than the parent). The piggyBAC mutants PB-60 and -65 are also of interest because of their high gametocyte production paired with slow (PB-60) or normal (PB-65) asexual growth. Perhaps the disrupted genes in these mutants impact the conversion pathway at different points, one that bypasses the cycle length and proliferation controls and one that does not.

3.5. Chapter Discussion

Malaria microvesicles are strongly gametocyte-inducing extracellular vesicles which \textit{P. falciparum} parasites release in significant amounts. Our findings indicate they are likely the carrier or one of the carriers of parasite density information in vitro. Thus they can explain previous observations that gametocyte production varies in response to parasite density in vitro and over the course of a continuous culture experiment.

\textit{Comparison with findings of a related paper}

Similarly, Regev-Rudzki et al. (2013)\textsuperscript{81} showed that plasmid DNA and drug resistance can transfer between parasite cells in co-culture, likely via extracellular vesicles believed to be exosomes, and also found that transferred vesicles stimulate gametocyte production. This paper reinforces our finding that extracellular vesicles allow cell-cell communication which
impacts a population’s allocation to the sexual stage. These researchers saw a 17fold increase in gametocytes correlated with drug treatment and vesicle-mediated plasmid transfer, comparable to our observation of 15fold induction, although theirs occurred over a longer time span. Regev-Rudzki et al. also found that the parasite protein PfPTP2 is required for the release of the plasmid-carrying vesicles they observed. Taken together, the papers show that malaria extracellular vesicles contain both host and parasite proteins and are capable of carrying nucleic acids, but the active component by which they trigger conversion is not known.

In contrast with our findings, the vesicles responsible for the plasmid transfer observed by Regev-Rudzki and colleagues are smaller than 120 nm. Additionally, the episomal plasmid transfer they observed occurs more efficiently in ring stage parasites, while our data (also see Chapter 4 and Appendix) shows strongly that release of the active factor in CM and of iMVs both occur in the final 1/3 of the cycle. Since iMVs are at least somewhat persistent it is possible that their uptake occurs in rings as well as in trophozoites and schizonts of the previous cycle. Alternatively parasites could release two populations of vesicles with different dynamics and possibly different biological activity.

_in vivo relevance._ Could the iMV-mediated cell-cell communication we observed be relevant to malaria parasites’ habitat within the human body? We report the visualization of multiple MVs being formed or released from an infected RBC at one time\textsuperscript{77}, and I detected a significant quantity of MVs directly in conditioned medium. CM and MVs maintain their in vitro effectiveness for at least 10 days at 4°C. Thus iMVs could likely reach high enough densities in the human bloodstream to impact developmental decisions within the parasite population. In
particular, iMVs could reach very high densities in microenvironments such as the bone marrow in which parasites sequester⁵.

In a human infection, the MV dynamics would certainly be more complex, and would differ in at least the following ways: 1. Hematocrit is 35-50% in humans as opposed to the 4% hematocrit used in our experiments; parasites potentially reach much greater actual density at the same parasitemia. Parasitemia in patients is often lower than in these experiments but can be >10% in hyperendemic areas⁶². 2. Macrophages take up iMVs⁷⁷; iMVs could potentially be lost in other ways. 3. Parasite population size changes over the course of an infection in a complex manner and depends on immune and other characteristics of the host. 4. The parasites that release iMVs according to our findings are late trophozoites and schizonts and thus will be sequestered in the microvasculature and organs. The distribution of parasites in the body could matter greatly for perceived “density”, so sequestration and whether MV uptake plays into the conversion decision before or after parasites sequester is an important question.

MVVs could provide a mechanism by which population information alters the switch rate by acting as a proxy for parasite density in the bloodstream or a local microenvironment such as bone marrow. This density-dependent behavior is reminiscent of quorum sensing which occurs in some bacteria and eukaryotic microbes, but differs in that a subset of the population instead of the entire population makes the switch at once. Small molecules and peptides are the most well defined as quorum sensing signals and have been identified in several eukaryotic communication systems. Interestingly, bacterium Pseudomonas aeruginosa has been shown to package its well-studied quorum sensing molecule, pseudomonas quinolone signal (PQS) in membrane vesicles for delivery to conspecifics⁸³. Microvesicles and exosomes from cancer cells
often contain proteins, microRNAs and mRNAs in their cargoes which are likely delivered to neighboring cells. Malaria microvesicles could be a means of packaging and delivering information in the form of small molecules, peptides or RNAs that trigger a signaling pathway leading to conversion.

*Environmental sensing and allocation to sexual vs. asexual development.* In section 3.4 I showed an inverse relationship between the parasite production and conversion rate of isogenic mutants of NF54, indicating that at least in this format, conditions that impair total proliferation also stimulate gametocyte production. In Chapter 4 we report that conditioned medium treatments that reduce parasite growth to about 30 or 35% of its maximum are also effective in stimulating conversion rates very strongly in the strain Pf2004. Many protocols for maximum gametocyte production in vitro involve culturing a fast-growing healthy culture until it reaches a high parasitemia in order to stress the cells, then rescuing the cells by dilution or addition of fresh medium so that healthy gametocytes can develop (e.g. 71). In a 2003 report Dyer and Day propose a model in which diffusible factors from low parasitemia cultures both suppress gametocyte production and slightly enhance asexual parasite growth, but diffusible factors from high parasitemia cultures strongly suppress parasite growth and enhance gametocyte production; this could lead to a burst of gametocyte production when an in vitro culture is in the log phase of growth especially just before the population plateaus. In vivo this could speculatively lead to bursts of gametocyte production towards the end of each population peak and shortly before the host immune response or other changes drastically restricts the asexual population.
A wide array of stimuli and environmental conditions have been reported to impact the sexual conversion decision in laboratory cultures, and some have also been reported from human or rodent infections. These many stimuli, however, would need to be processed and integrated by one or a few mechanisms and eventually converge on activating AP2-G and other known early gametocyte genes (see Model, Figure 19). My results along with previous reports suggest that gametocyte-inducing stimuli likely fall into two groups: 1. Specific stimuli that target molecular components of the conversion pathway. This could include cAMP, 8-bromo-cAMP, possibly cholera toxin, phorbol esters, and microvesicles. 2. Stressful or damaging stimuli that restrain asexual growth to about 1/3 of its maximum or less. This would include for example nutrient limitation, certain antimalarial drugs, toxins in red cell lysate, and toxic molecules such as diminazen (Berenil). Detection of these potentially lethal threats could occur through detection of internal changes in the cell by mechanisms that feed into the sexual conversion pathway and eventually activate AP2-G. The observed differences in gametocyte production between strains could be partially due to their different growth rates and partially due to the sensitivity of their conversion machinery to environmental challenges.

*Future questions: targets of iMVs and variations in iMV production or sensitivity.*

Questions of interest for future research include the active components of iMVs, how they are internalized by parasite cells, and how they feed into the conversion pathway (Model, Figure 19). Additionally, there could be differences in MV production or MV sensitivity among strains with different tolerance to high parasitemia and different gametocyte production behavior, such as between strains recently adapted to culture and long term cultured strains.
The Marti Lab has planned potential experiments to investigate the targets of MV signaling. Flow sorting experiments will be performed to separate parasites from the same population which have taken up labeled MVs and those which have not, and transcriptional profiling will be performed on the two populations. Flow sorting of GFP-expressing parasites has already been established in the Marti Lab in collaboration with Dr. Natasha Bartaneva\textsuperscript{84}. These experiments could help us discover unknown components of the gametocyte conversion pathway that are directly downstream of iMV uptake.

In order to attain a full understanding of sexual conversion, malaria researchers will need to understand how the reported stressful stimuli are integrated into the asexual vs. sexual cell fate decision. This will help the malaria community to understand how parasite populations interact with one another and how gametocyte and parasite growth dynamics could interact in human infections. In turn this can lead to a better understanding of how our interventions direct parasite populations toward or away from gametocyte production.

**Materials and Methods**

3D7/0164-GFP parasites or 3D7/0744-GFP parasites with gametocyte-specific GFP expression\textsuperscript{23} were used in all experiments except those in Figure 13. CDPKS-DD parasites were obtained from the laboratory of Dr. Jeffrey Dvorin (Childrens Hospital Boston). PB-60 parasites (Figure 13a) and the piggyBAC library strains (Figure 13b) were obtained from the laboratory of Dr. John Adams (University of South Florida). Microvesicles for Figure 12 were purified from non-fluorescent 3D7 and CS2 parasites as described\textsuperscript{77}. Parasites were cultured as described in Chapter 2, except that malaria culture medium with 10% human serum was used for all
experiments. Additionally, for transgenic strains 3D7/0164-GFP and 3D7/0744-GFP 4 nM WR 99210 (Jacobus Pharmaceuticals) was added to the medium to maintain plasmid selection. Flow cytometry to quantify gametocytes and total parasites was performed on a Beckman Coulter Quanta SC flow cytometer as described in Chapter 2. 10 kDa cellulose acetate molecular weight cutoff filters (Centrisart, Sartorius Stedim) were used for Figure 11c.
Chapter 4: An assay to probe *Plasmodium falciparum* growth, transmission stage formation and early gametocyte development

**Note:** The following is a manuscript version that was submitted to *Nature Protocols* in February 2015. The article is currently in revision as of March 30th 2015. Formatting and figure and section numbering were altered for this dissertation.

**Author contributions to manuscript:** I. Goldowitz wrote the first draft of all manuscript sections except figure legends, figures and Anticipated Results, and N. Brancucci created the figures and wrote the first draft of the figure legends and Anticipated Results section, with input from M. Marti. I. Goldowitz, N. Brancucci, and M. Marti revised the entire manuscript and all authors contributed to revisions before submission.
An assay to probe *Plasmodium falciparum* growth, transmission stage formation and early gametocyte development

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Abstract

Conversion from asexual proliferation to sexual differentiation initiates the production of gametocytes, the malaria parasite stage required for human-to-mosquito transmission. The presented protocol describes a high throughput-compatible assay designed to probe the effect of drugs or other perturbations on asexual replication, sexual conversion and early gametocyte development in the major human malaria parasite Plasmodium falciparum. Synchronized asexually replicating parasites are induced for gametocyte production by addition of conditioned medium and exposed to the treatment of interest during sexual commitment or at any subsequent stage of early gametocyte development. Flow cytometry is used to measure asexual proliferation and gametocyte production via DNA dye staining and the gametocyte-specific expression of a fluorescent protein, respectively. This screening approach may be used to identify and evaluate potential transmission-blocking compounds and to further investigate the mechanism of sexual conversion in malaria parasites. The full protocol can be completed in 11 days.

4.1. Introduction

Following injection by a mosquito into a human host and a single round of mitotic expansion within hepatocytes, malaria parasites undergo an indefinite number of asexual replication cycles within red blood cells (RBC). During each of these intra-erythrocytic developmental cycles (IDC), a small proportion of parasites stop replicating and initiate development into sexual precursor cells in a process known as sexual conversion. These sexual
precursor cells, called gametocytes, are at maturity the only parasite form capable of establishing an infection in the mosquito vector. Sexual conversion and proper gametocyte development are thus essential for transmission of malaria. The assay described here focuses on *P. falciparum*, the parasite species responsible for the vast majority of the more than 600,000 malaria deaths each year. The presented assay protocol allows experimenters to manipulate the rate at which *P. falciparum* parasites commit to either the asexual or the sexual developmental pathway in a highly targeted and reproducible manner. The assay enables the screening of drugs or other perturbations of interest for their effect on (i) asexual parasite replication, (ii) early gametocyte development and (iii) the rate at which parasites differentiate into gametocytes (i.e., the sexual conversion rate).

Strategies for preventing transmission are essential for efforts aiming at malaria elimination and eradication. As gametocytes are required for transmission and are produced in low numbers, they represent an important and potentially vulnerable target for transmission blocking therapies. However, most antimalarial drugs in current use perform poorly at killing gametocytes and thus are unable to fully prevent malaria transmission. Some reports have suggested that treatment with antimalarial compounds including pyrimethamine, chloroquine and mefloquine may even increase the number of gametocytes produced in patients or under *in vitro* conditions, possibly by increasing sexual conversion rates. Similarly, enhancement of gametocyte production and transmission has been observed with drug resistant parasites in rodent infections. Clearly, there is an important but unmet need for methods to identify agents that inhibit gametocytes throughout all stages of development.
Additionally, better methods are required to ensure that future antimalarial drugs do not stimulate gametocyte production.

We previously developed a high throughput-compatible drug screening assay\textsuperscript{23}, which was the first assay capable of targeting both sexual conversion and the earliest stages of gametocyte development. The assay involves the simultaneous measurement of total parasite proliferation and gametocyte production during a single blood-stage replication cycle. We have used variations of this assay to identify an environmental stimulant of gametocyte conversion\textsuperscript{90} and to complete dose-response and window-of-action analyses of antimalarial compounds under development\textsuperscript{91,92}. Here we present an updated and streamlined version of the protocol (Figure 14) that enables screening of drugs or other perturbations against highly synchronous early gametocytes. It additionally allows experimenters to interrogate the biology of sexual conversion and to test antimalarial compounds under development for potential inhibitory or stimulatory effects on gametocyte production.

*Stage conversion and transmission of P. falciparum parasites.* Recent discoveries have begun to shed light on the mechanisms governing the production of gametocytes in *Plasmodium*, showing that environmental, genetic and epigenetic factors can drive sexual conversion. For example, recent studies have identified a transcription factor, AP2-G\textsuperscript{14,15}, as well as the epigenetic factors PfHP1 and PfHDA2\textsuperscript{93,21} as regulators of sexual commitment. In addition, it was shown that parasites use microvesicle-mediated cellular communication that appears to modulate the rate at which sexual commitment is initiated\textsuperscript{81,94}. Finally, various cellular components have been reported to be required for sexual conversion, including environmental sensing and signaling molecules\textsuperscript{56} (i.e. receptors, kinases, and G proteins\textsuperscript{64,95,65}}
cell cycle control proteins, as well as cellular stress responses \(^{63,97}\). Through an as yet unknown pathway, external stimuli are believed to manipulate cell fate decision already in late stage asexually replicating parasites (schizonts). These schizonts commit to either the sexual or the asexual cell fate \(^{98}\) and their progeny will hence either develop into gametocytes or continue asexual proliferation. Altogether these data suggest that multiple layers of regulation act in concert in order to induce the production of gametocytes. Interestingly, the protein classes suggested to be involved in \(P. falciparum\) sexual conversion include numerous major drug targets in other eukaryotes. Identifying the factors associated with conversion in \(P. falciparum\) is therefore likely to uncover promising targets for future transmission-intervention strategies.

\emph{Comparison with other published \(P. falciparum\) in vitro high throughput assay protocols.}

A number of high throughput-compatible whole cell assay methods have been developed and applied to screen compound libraries against \(Plasmodium\) asexual and sexual blood stages, as well as liver stage parasites. Existing screens to identify compounds with an effect on sexual development generally focus on gametocytes in the second half of the 8 to 12-day maturation process \(^{99-102}\). For example, Peatey et al.\(^{100}\) and Sanders et al.\(^{102}\) recently screened compounds against pure populations of mature sexual stage parasites by using ATP bioluminescence or the gametocytes’ ability to exflagellate, respectively, as a readout. In contrast, D’Alessandro and colleagues\(^{103}\) assessed viability of purified mid-stage gametocytes in a lactate dehydrogenase assay, while Lucantoni et al.\(^{101}\) screened against early to mid-stage gametocytes using a luciferase reporter-based system.
Screens targeting young gametocytes are largely underrepresented, which is at least partially attributable to the fact that these stages are difficult to separate from the bulk of asexually replicating parasites. As we demonstrate with the presented assay, flow cytometry combined with an appropriate reporter system can make such screens possible. Other than the presented screen, Wang et al. developed the only cytometry-based approach that enables targeting sexual stage parasites, including early gametocytes\(^\text{104}\). In that assay, drug susceptibility of developing gametocytes is tested after they have been purified from asexual cells by sequential treatments with sorbitol, Percoll and heparin. Our protocol, by contrast, uses flow cytometry detection of a gametocyte-specific fluorescent reporter to quantify early gametocytes within the asexual population, and thus avoids the necessity of purifying gametocytes or applying compounds that specifically kill asexual cells. The assay, hence, places special emphasis on the controlled induction of gametocytogenesis. The improved protocol allows experimenters to reproducibly generate either (i) asexual parasite populations containing negligible numbers of gametocytes or (ii) cultures that are greatly enriched in highly synchronous gametocytes – a feature that is unique to this screen. This allows screening for compounds that inhibit sexual conversion and/or development as well as for conditions that trigger gametocytogenesis. Consequently, diverse questions about drug susceptibility and the biology of sexual differentiation may be addressed using the same basic and simple assay.

Due to its excellent performance in inducing highly synchronous gametocyte populations and in contrast with most published screens, the presented assay does not require physical purification of sexual stage parasites or removal of gametocytes formed during previous IDCs. Further, the effect of the perturbation of interest on asexual parasite
proliferation is detected in the same well, making the assay ideal for screens to identify molecules that specifically target gametocytes but not asexual blood stages and *vice versa*.

4.2. Experimental design

In the presented assay, modified from Buchholz et al.\textsuperscript{23}, stage synchronized blood-stage parasites are seeded in 96-well plates (220μl per well, 0.3% parasitemia, 2.5% hematocrit (HC)) at 24-32 hours post invasion (hpi) (IDC-IV, Figure 14). In wells to be stimulated for high gametocyte production, sexual conversion is induced at that time point by the addition of parasite-conditioned medium (CM) for 24 hours under well-defined conditions. The CM is produced on a separate parasite culture maintained at high parasitemia (IDC-III, Figure 14).

If screening for inhibitory effects on sexual conversion and/or gametocyte development, the culture is induced by applying CM and test compounds are simultaneously added to the cells or at any later time point, depending on the experimenter’s requirements. If screening for stimulatory effects on sexual conversion, cells are not induced prior to treatment with test compounds. In a set of control wells, parasites are cultured without drug addition. In presence of CM, these will produce high proportions of gametocytes, whereas only few gametocytes are formed under non-inducing control conditions. After the parasites have re-invaded erythrocytes, flow cytometry is first used to determine parasitemia (IDC-V, 24-32hpi, Figure 14) and, 38 hours later, to evaluate the proportion of early gametocytes (IDC-V, 62-70hpi, Figure 14) using DNA dye staining and fluorescent protein expression, respectively. The effect of drug treatment on the proportion of parasite offspring that become gametocytes (referred to as the
conversion rate), on the overall asexual parasite proliferation rate, and/or on gametocyte survival, are determined by comparison to the high-gametocyte and low-gametocyte control wells (see Procedures).

Gametocyte detection relies on expression of the red fluorescent reporter tandem Tomato (tdTom), driven by the promoter of gametocyte-specific gene etramp10.3 \((PF10_0164/PF3D7_1016900)\) \(^{105,32,33}\). Fluorescence intensity of gametocytes produced in parasite lines carrying this reporter system (termed 164-tdTom) allows for stage-diagnostic signal detection by flow cytometry after 62hpi. At this time point, gametocytes that may have formed during the next IDC are not yet detectable \(^{105}\).

As described above, multiple assay setups are available to address different questions. Drugs or other perturbing conditions can be evaluated for negative effects on sexual conversion or early gametocyte development as well as for effects that potentially induce or enhance the production of gametocytes. As the assay does not require purification of gametocytes, the impact of treatment on asexual parasites can be measured simultaneously, allowing to investigate differences in drug susceptibility between asexual and sexual stage parasites in the same well.

**Optimization.** On the basis of our published protocol\(^{23}\) we have streamlined and updated the assay setup. For simplicity and ease of use with large culture volumes, we replaced the combination of sorbitol and Percoll synchronization steps\(^{23}\) with a series of four sorbitol treatments (IDC-1 through IDC-IV, Figure 14), generating parasites synchronized to an 8-hour age window. The presented assay is now based on the gametocyte-specific expression of a tdTom reporter. Compared to the former protocol\(^{23}\), in which we used GFP-fluorescence
**Figure 14. Flowchart depicting individual steps of the protocol.** The timeline represents consecutive parasite developmental cycles (IDCs). **IDC-I:** Parasite cultures are sorbitol-treated twice to synchronize the population to an 8-hour age window. **IDC-II:** The population is split into lower (LP) and higher parasitemia (HP) cultures containing below 1% and 1.5%-2% infected red blood cells, respectively. **IDC-III:** The LP culture is sorbitol-synchronized at 0-8hpi. The HP culture is adjusted to a parasitemia of 5.5%-6.5% at 24-32hpi and incubated for 16 hours before collecting the conditioned medium (CM) by three centrifugation steps. **IDC-IV:** The LP culture is sorbitol-synchronized at 16-24hpi and adjusted to a parasitemia of 0.3%. At 24-32hpi, the cells are washed and resuspended in 90% or 95% CM (complemented with complete media) and seeded to wells of a 96-well plate. These conditions will induce sexual conversion in a high proportion of forming schizonts and the compound to be screened may be added at this or at any following time point of the protocol (indicated by a dashed arrow). Parasites cultured in absence of the compound and under non-inducing conditions, i.e. with complete media, serve as positive and negative controls, respectively. **IDC-V:** After the parasites have re-invaded fresh erythrocytes (0-8hpi), the culture media is exchanged by complete media. The media is henceforth exchanged daily. At 24-32hpi, parasitemia is determined by flow cytometry using an aliquot of SYBR Green-stained cells. At 62-70hpi, young gametocytes have acquired enough tdTom-fluorescence for cytometry-based quantification.
Work Flow

Timeline

<table>
<thead>
<tr>
<th>0-8hpi</th>
<th>16-24hpi</th>
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<tr>
<td>IDC-I</td>
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<td>IDC-II</td>
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<td>IDC-III</td>
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<td>IDC-IV</td>
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<td>0-8hpi</td>
<td>24-32hpi</td>
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<td>IDC-V</td>
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<tr>
<td>&gt;62-70hpi</td>
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- PREPARATION OF EXPERIMENTAL CELLS
- SORBITOL-SYNCHRONIZE TO 8 HOUR TIME WINDOW
- CONDITIONED MEDIA (CM) PREPARATION
- SPLIT TO LOW PARASITEMIA
  - keep parasitemia below 1%
  - RE-SYNCHRONIZE to ensure synchronicity
- SPLIT TO HIGH PARASITEMIA
  - adjust parasitemia to 1.5%-2%
  - COLLECT CM PRIOR TO RE-INVASION
  - spin 3x to remove cells
  - store at 4°C
- SYNCHRONIZE, WASH AND APPLY CM
  - DILUTE CM TO WORKING CONC.
  - 90% CM 95% CM (control)
- EXCHANGE MEDIA 24 HOURS LATER
- DETERMINE PARASITEMIA BY FLOW CYTOMETRY
- DETERMINE GAMETOCYTE NUMBERS BY FLOW CYTOMETRY

Figure 14. (Continued)
as a readout, this has significantly improved the signal to noise ratio (from below 10 to 1142 ± 186 SD) by avoiding an overlap between the spectrum of our reporter and the autofluorescence of erythrocytes in green wavelengths. Moreover, we found *P. falciparum* strain Pf2004 to be favorable for the purposes of our assay. In comparison to reference isolates, Pf2004 is a more recently culture-adapted, gametocyte-producing isolate from Ghana. Compared to other laboratory strains such as 3D7 or HB3, the Pf2004/164tdTom line is more responsive to environmental stimuli that induce sexual conversion (see also Figure 15a), while the rate at which gametocytes are formed under normal growth conditions (i.e. baseline conversion rate) is negligible. The assay thus allows for the controlled production of highly synchronous gametocyte populations that can be perturbed as soon as sexual commitment is initiated.

Although we provide a protocol for the reproducible induction of gametocytogenesis, sexual stage conversion remains a multilayered process sensitive to a variety of both environmental and parasite-intrinsic factors. Consequently, gametocyte conversion rates vary between different inductions, depending on the exact composition of the experimental cell population and the batch of CM used (referred to as an “induction batch”). Z’-factors within individual induction batches consistently remain above 0.7 (0.83 ± 0.07 SD), demonstrating the high quality of the assay. Z’-factors were calculated according to Zhang et al., using induced Pf2004/164-tdTom cultures treated with lethal doses of DHA as negative and non-treated cells as positive controls. In contrast to other screens, where the dynamic range of the signal is defined by technical constraints, biological variability is an inherent component of the presented assay and therefore, Z’-factors calculated from across induction batches fail to
Figure 15. Cytometric readout of gametocyte production and survival. A. Bar graphs quantifying the number of sexual cells produced under gametocyte-inducing and control conditions for cell lines 3D7/164-tdTom and Pf2004/164-tdTom (mean ± SD). The time point of flow cytometry-based quantification is indicated in hpi. Live cell images are shown for Pf2004/164-tdTom gametocytes and illustrate tdTom reporter expression (red) at 62-70hpi and 86-94hpi. B. Example of cytometric scatterplots quantifying SYBR Green (x-axis) and tdTom (y-axis) fluorescence for Pf2004/164-tdTom parasites cultured under control (left plot) and gametocyte-inducing (right plot) conditions. Flow cytometry was carried out at 86-94hpi. Corresponding gametocyte conversion rates are indicated and were calculated according to step 21 of the presented protocol (see Procedures).
**A Gametocyte Induction**

**Pf3D7/164-tdTom**

**Pf2004/164-tdTom**

**B Cytometry-Based Gametocyte Quantification**

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**Figure 15. (Continued)**
describe the screens’ performance. To prevent false positive and/or negative hits, screening results must thus always be interpreted in relation to ‘induced’ (i.e. treated with CM) and ‘non-induced’ control conditions from the same induction batch (see Procedures). Compared to the consistently low conversion rates of non-induced populations (0.3% ± 0.06% SD), CM-treated Pf2004/tdTom-164 cultures produce at least 40 (61.8 ± 17.8 SD) times more gametocytes, typically converting to the sexual pathway at a rate of 14% to 24% (17.8% ± 5.4% SD). This high responsiveness of the Pf2004/tdTom-164 line to CM-treatment is particularly important when screening compounds or growth conditions that induce gametocytogenesis, and generally allows one to identify hits with high accuracy.

Although we recommend the use of the Pf2004/tdTom-164 line, the assay can also be carried out using transgenic 3D7 parasites, as reported in our original screen\textsuperscript{23}, or using HB3 parasites carrying the respective reporter plasmid. Higher baseline conversion rates of both of these strains compared to Pf2004, however, may limit the ability to detect perturbations that stimulate sexual conversion. Transgenic 3D7, HB3 and Pf2004 strains can be requested from our laboratory and are available with GFP or tdTom reporters under control of the gametocyte-specific promoters of PF3D7_1016900, PF3D7_1477300 or PF3D7_1477700. In addition, the assay should be adaptable for screening of non-transgenic parasites using immunolabeling of gametocytes as readout.

Limitations. Although we recommend that personnel performing this screen have experience in \textit{P. falciparum in vitro} culture, the individual steps of the assay are otherwise relatively simple to carry out. As compared with plate reader-based methods, the flow cytometry readout of our assay places a limit on the number of compounds that can be
screened per unit of time. Labor is a limiting factor of the presented assay because of the multiple synchronization and readout steps as well as the long time span of the full assay (Figure 14), and it can be beneficial to have two individuals share steps in the protocol. Because multiple cell cycles are required for sexual conversion and gametocyte development to a distinguishable stage, assays that screen gametocytes, whether mature or immature, necessarily occur over a longer time span than assays targeting asexual blood stages. When developing the original assay, we observed highly increased gametocyte levels after treatment with sublethal doses of some antimalarials such as atovaquone and methylene blue\textsuperscript{23}. Further investigation of this phenomenon revealed that the cytostatic effect of these drugs results in an immediate but transient faux activation of the fluorescent reporter preceding reporter expression during early gametocyte development. Importantly, due to the temporal differences in reporter activation, the presented assay is capable of distinguishing these ‘faux induced’ parasites from sexually committed cells (see Figure 16 and discussion in the Anticipated results section).

4.3. Materials.

Reagents

\textit{P. falciparum} line Pf2004/164-tdTom, tested for gametocyte production (Marti Lab; alternative strains and fluorescent reporters are available)

\textbf{CAUTION} \textit{P. falciparum} is a human pathogen; consult local regulations for handling and waste disposal requirements. Wear gloves and a lab coat.
**Morphology of CM-producing parasites**

![Morphology of CM-producing parasites](image)

**Figure 16. Conditions during CM production hamper parasite development.** Diff-Quick stained blood smears show morphology of cells used to produce CM. Smears were made at the time point of CM collection (40-48hpi). Parasitemia is indicated. Note that the development of CM-producing parasites is delayed compared to control cells kept at a low parasitemia. In the assay, highest rates of sexual conversion were achieved by using CM produced from cells at a parasitemia of 5.6% (data not shown).
Human red blood cells, type O+ (Research Blood Components or local vendor)  *CRITICAL

Erythrocytes must always be provided fresh (i.e., <10 days old).

!CAUTION Blood cells may contain human pathogens; consult local regulations for handling and waste disposal requirements. Wear gloves and a lab coat.

Malaria Culture Medium components:

Distilled water (e.g., from Millipore distilled water system)

RPMI 1640 with phenol red (Sigma; Cat. no.: R6504),

HEPES free acid (Calbiochem/EMD Milli; Cat. no.: 391338)

Gentamycin (Sigma; Cat. no.: G1397)

Human serum (Interstate Blood Bank or local vendor). Store at -20°C until immediately before use.

!CAUTION Human serum may contain human pathogens; consult local regulations for handling and waste disposal requirements. Wear gloves and a lab coat.

Hypoxanthine (Sigma; Cat. no.: H9377)

NaHCO₃ (Sigma; Cat. no.: S5761)

Flow cytometry buffers and calibration beads (Miltenyi Biotec or appropriate for your flow cytometer):

Running buffer (Miltenyi; Cat. no.: 130-092-747)

Washing solution (Miltenyi; Cat. no.: 130-092-749)

Storage solution (Miltenyi; Cat. no.: 130-092-748)

MACSQuant Calibration Beads (Miltenyi; Cat. no.: 130-093-607)
Tissue culture consumables:

Glass slides (VWR; Cat. no.: 16004-368)

Sterile filter, 0.22μm pore size (Thermo Fisher/Nalgene; Cat. no.: 569-0020)

Sterile 20μl, 200μl, 1ml tips (Denville; Cat. nos.: P1121, P1122, P1123)

Sterile 15ml and 50ml Falcon tubes (Corning; Cat. nos.: 430791, 430829)

Sterile TC treated 96-well plates, flat bottom (Corning; Cat. no.: 353072)

Sterile 100x15mm and 150x15mm Petri dishes (Corning; Cat. nos.: 351029, 351058)

Sterile 5ml, 10ml, 25ml, 50ml pipettes (VWR; Cat. no.: 89130-896, -898, -900, -902)

Sterile 250ml media bottles (Thermo Fisher/Nalgene; Cat. no.: 2019-0125)

WR 99210 (Jacobus Pharmaceuticals)

Gas mix for malaria culture 5% CO₂, 1% O₂, balance N₂ (Med-Tech Gases or local vendor)

Diff-Quik, Giemsa-based or similar slide staining system:

Methanol (EMD; Cat. no.: MX0475P)

Hemacolor Solution II (EMD; Cat. no.: 65044B-85)

Hemacolor Solution III (EMD; Cat. no.: 65044C-85)

SYBR Green (Lifetech; Cat. no.: S7567)

!CAUTION SYBR Green binds DNA and may be a mutagen .

Sterile Phosphate Buffered Saline (PBS) (Lonza; Cat. no.: 17-517Q)

Small Molecule Libraries

!CAUTION Some molecules in the library may be toxic.

Dimethyl Sulfoxide (Sigma; Cat. no.: D2650)

Sorbitol (Calbiochem; Cat. no.: 56755)
**Equipment**

Light microscope (e.g. Olympus CX21) for viewing stained slides

Biosafety level 2 rated (Level II A2) tissue culture cabinet (e.g. Nuare Model NU-425-400)

Tissue culture incubator maintained at 37°C (e.g. Forma Scientific, Model 3110)

Culturing chamber (e.g. Billups-Rothenberg; Cat. no.: MIC-101) or gassed incubator system

Pipetboy (e.g. Integra; Cat. no.: 155000)

Tabletop centrifuge (e.g. Eppendorf 5810R)

pH meter (e.g. Mettler Toledo SevenEasy)

>4l glass flask

Water bath at 37°C

Flow cytometer (e.g. MACS Quant VYB, Miltenyi Biotec). The flow cytometer used must be able to excite and detect both SYBR Green and TdTomato fluorescence and must be compatible with 96-well plates.

FlowJo (Tree Star) or similar flow cytometry analysis program

**Reagent setup**

Parasite cell preparation: For thawing, freezing and maintenance of *P. falciparum* cells, refer to Methods in Malaria Research. Pf2004/164-tdTom parasite cultures are kept at 37°C in MCM containing 4nM WR99210. Media is to be exchanged daily. Unless otherwise stated, the culture hematocrit is kept at 5%. Cultures are maintained in sterile 100x15 mm Petri dishes (10ml culture volume per dish) or 150x15 mm dishes (30ml culture volume per dish). We recommend
minimizing handling time outside ideal growth conditions (37°C in gas mixture optimized for malaria culture). After each step involving handling parasites, promptly return dishes to a culturing chamber and add optimized gas mixture, then return chamber to an incubator at 37°C. Alternatively, return to a gassed TC incubator system at 37°C.

Per screening plate, start the experiment with ~10ml of culture at a ring stage parasitemia of at least 2%. To evaluate parasitemia (percentage of parasite-infected red blood cells) place 5μl culture suspension on a glass slide and use the edge of another glass slide to smear. Stain using Diff-Quik or another staining system. Count infected cells per 2000 total RBCs using a light microscope.

!CAUTION P. falciparum is a human pathogen; consult local regulations for handling and waste disposal requirements. Wear gloves and a lab coat.

*CRITICAL To maintain sterility of cells, all steps involving cells that will be returned to culture, as well as culture reagent preparation, must be carried out under sterile conditions in a biosafety cabinet.

*CRITICAL P. falciparum lines can lose the ability to produce gametocytes after extended in vitro culturing 26. Keep multiple aliquots of the original parasite stabilate in liquid nitrogen and maintain gametocyte-producing cultures at low passage number.

Prepare incomplete malaria culture medium: In clean and sterile flask, prepare 4l of medium as follows: Dissolve 41.76g RPMI powder with phenol red and 23.76g HEPES (free acid) in 2.8l ddH2O and stir for at least 30min. Separately, add 400mg hypoxanthine to 80ml boiling water and mix to dissolve. Add 40ml of the hypoxanthine and 1l ddH2O to the 4l flask. Use a pH meter
to adjust pH to exactly 6.75 with 1M NaOH. Filter sterilize by passing through a filter with 0.22μm pore size in a tissue culture hood, and store at 4°C for up to four weeks.

Prepare complete malaria culture medium (MCM): Dissolve NaHCO$_3$ to 3.6% (w/v) in water and filter sterilize. To 210ml incomplete malaria culture medium, add 14ml NaHCO$_3$ solution and 25ml human serum under sterile conditions. Mix and store at 4°C for up to two weeks.

Prepare 5% sorbitol (w/v) in distilled water. Filter sterilize by passing through a 0.22μm filter in tissue culture hood and store at 4°C for up to 6 months. Sorbitol treatment lyases erythrocytes infected with 24-48 hour old $P. falciparum$ cells, and thus synchronizes the parasites to 0-24hpi.

Prepare SYBR Green staining solution in PBS immediately before use. Dilute SYBR Green stock solution 1:5000 in PBS.

Prepare aliquots of WR 99210: Completely dissolve WR 99210 to 2mM in 10ml RPMI-HEPES in a 15ml falcon tube by rotating for 1-3 hours. Sterile filter and store this stock solution at -20°C. To prepare working solution, dilute stock 1:20 (to 10μM) in RPMI-HEPES and store at 4°C for up to four weeks. Add 4μl of working solution per 10ml of parasite culture.

**Equipment setup**

Flow cytometer setup: Regularly run calibration and cleaning protocols on your flow cytometer according to manufacturer’s instructions. On the MACS Quant VYB, tdTomato is detected in the
615/20 channel (Y2) and SYBR Green is detected in the 525/50 channel (B1). For both parasitemia readout (step 17) and gametocytemia readout (step 19), optimized settings on the MACS Quant VYB are as follows:

- **FSC channel (Trigger on this channel): 270 volts**
- **Y2 channel: 375 volts**
- **B1 channel: 270 volts**

Low flow rates are used.

If an alternate flow cytometer is used, the specific settings may need to be optimized to ensure good separation between the uninfected RBC, asexual parasite, and gametocyte populations.

### 4.4. Procedure.

A flow chart of the assays’ setup is shown in Figure 14.

The following procedure describes the parallel generation of enough CM and experimental cells for 180 gametocyte inductions in the 96-well format. If the assay is scaled up, it may be advisable to perform the labor-intense production of CM in advance. CM can be kept for at least 10 days at 4°C.

The standard assay as provided below is designed to screen compounds for inhibitory effects on gametocyte production and development, as well as on asexual proliferation. The compounds of interest are hence added simultaneously with the gametocyte-inducing CM or at any later time point, depending on the question asked. Alternatively, using the same assay setup, compounds or changes in culture condition can be probed for a positive effect on the
rate at which parasites switch to the sexual pathway. If screening for such gametocyte-enhancing effects, non-induced parasite are probed instead, whereas gametocyte-induced cultures serve as positive control. See step 12.

**IDC-I**

**First sorbitol synchronization**

1) Pre-warm MCM and sorbitol solution to 37°C in a water bath.

2) Evaluate parasitemia of a 30ml (asynchronous) parasite culture by light microscopy using Diff-Quik or Giemsa stained blood smears (see Reagent setup).

Note: Only start the protocol if more than 2% of the erythrocytes are infected with early ring stage parasites.

3) Pellet cells in a 50ml conical centrifuge tube, discard supernatant and resuspend the cells in 8 times the pellet volume of sorbitol solution. Pipet up and down to mix and incubate at 37°C for 5-10 minutes. Wash cells once in 10ml pre-warmed MCM and resuspend the culture in 30ml of pre-warmed MCM.

Note: Sorbitol treatment synchronizes the parasites to a 24-hour time window (0-24hpi).

**Second sorbitol synchronization**

4) 16 hours after the first sorbitol synchronization, repeat steps 1 through 3 to bring parasites to an 8-hour time window (16-24hpi).

**IDC-II**

**Preparation of experimental cells and cultures producing the conditioned medium**
5) Evaluate parasitemia after parasite re-invasion is completed.

Note: A minimum of 3% parasitemia is required.

6) Prepare a low parasitemia (LP) and a high parasitemia (HP) culture, which will be used for the generation of experimental cells and for the preparation of CM, respectively. *CRITICAL Dilute the LP culture to a parasitemia of ~0.3% and keep the proportion of infected cells below 1% to prevent stress-responses that may lead to a premature induction of gametocytogenesis. Bring the HP culture to a parasitemia of 1.5-2% and expand the culture volume according to your requirements (220μl of CM are needed per well of a 96-well plate; save an additional 110μl per well for the washing procedure given in step 12).

**IDC-III**

*Third sorbitol synchronization of experimental cells*

7) Re-synchronize early ring stage parasites (at 0-8hpi) of the LP culture by repeating steps 1 through 3.

*Collection of conditioned medium*

8) Adjust the HP culture to a parasitemia of 5.5-6.5% when parasites are 24-32hpi. Allow cells to develop for 16 hours to 40-48hpi.

*CRITICAL Parasites of the HP culture are used to produce the CM and, hence, should be placed in 100% fresh and pre-warmed MCM at this time point.

9) 16 hours after step 7, when parasites are at 40-48hpi, pellet cells (5min, 800g) in a conical centrifuge tube and collect the supernatant (this is the CM). Pellet once more to remove
remaining cells, collect the supernatant, and store the CM at 4°C for up to 10 days. You can discard the culture pellet.

Note: Culturing late stage asexual parasites of the line Pf2004/164-tdTom at a parasitemia greater than 5% may drastically affect their development. At the time point of CM collection, expect the parasites to deviate from the typical morphology of schizonts at 40-48hpi (Figure 16).

**IDC-IV**

*Fourth sorbitol synchronization of experimental cells*

10) Re-synchronize late ring stage parasites (at 16-24hpi) of the LP culture (as in steps 1-3) and accurately determine parasitemia by light microscopy or by flow cytometry. *CRITICAL* Dilute to a parasitemia of exactly 0.3% and expand the culture to the desired volume (110μl are needed per well of a 96-well plate).

Note: If the parasitemia or the culture volume is too low for your requirements, allow parasites to complete another IDC and repeat this step.

*Setting up the library screen*

11) Complement CM (collected in step 9) by adding MCM to a final concentration of 10% (v/v).

Mix and pre-warm this CM working solution to 37°C in a water bath. Optional: Prepare a second CM working solution containing 95% CM and 5% MCM.

*CRITICAL* Using CM working solutions at different concentrations may drastically affect gametocyte conversion rates of the experimental cells. Also, best conditions may vary between
different cell lines. In our hands, the 90% CM working solution produced best and highly reproducible results. It is advisable, however, to test different conditions before starting the screen. Alternatively, depending on practicability and size of the compound library, the screen may be performed using both 90% and 95% CM conditions.

12) Prepare experimental cells at 24-32hpi:

i. For cells to be treated with drugs (and untreated positive-control cells), induce gametocytes by preparing as follows: Pellet the required amount (110μl are needed per well of a 96-well plate) of the LP culture in a conical centrifuge tube and wash in 20 pellet volumes of the CM working solution. Resuspend the cells in 40 pellet volumes of the CM working solution.

ii. Prepare cells to be used as ‘non-induced’ negative controls as follows: Pellet the required amount (110μl are needed per well of a 96-well plate) of the LP culture in a conical centrifuge tube and wash the cells in 20 pellet volumes of fresh MCM. Resuspend in 40 pellet volumes of fresh MCM.

*CRITICAL If drugs are screened for effects that induce or enhance sexual conversion, prepare non-treated control cells as described in i. These conditions will control for maximal induction of sexual conversion. Prepare the experimental cells to be treated as described in ii.

13) Distribute 220μl of cell suspension per well of a 96-well plate. Fill unused surrounding wells with incomplete medium to prevent evaporation.

Note: the parasite culture now contains 0.3% infected erythrocytes at a HC of 2.5%.

14) Add the compounds of interest (in technical triplicates) now or at any later time point during sexual commitment/early gametocyte development and mix by pipetting up and down. Include untreated control wells in every plate. Do not add drug to these wells, but do include
the maximum concentration of compound vehicle (e.g. DMSO) used in the experiment.

*CRITICAL* Changes in culture volume may affect gametocyte conversion rate. We thus recommend adding relatively low and equal volumes (2-10µl) of compound stock solutions to each well. We observed that DMSO concentrations of up to 0.3% do not affect parasite growth or gametocyte production/development.

**IDC-V**

*Media exchange*

15) Pellet cells 24 hours after CM addition, remove 200µl media and resuspend in 200µl of pre-warmed MCM.

Note: The cultures now contain early developing gametocytes that are not yet morphologically distinct from asexual parasites. Depending on the setup of your experiment, you may want to add, re-add or remove the compounds of interest at this time point. In the latter case, remove the medium completely and wash 3 times in 200µl of pre-warmed MCM before incubating the cells in 220µl MCM.

**IDC-V (and early gametocyte development)**

*Measurement of parasitemia by flow cytometry*

16) At 24-32hpi, resuspend cells and transfer 10-20µl of each well to 100µl of SYBR Green staining solution (preloaded in a 96-well plate). Mix and incubate at 37°C for 20min. Protect from light. Pellet cells and wash twice in 200µl PBS. Resuspend in 200µl PBS.
17) Run flow cytometry to determine parasitemia. Measure 100’000 events per well. See Equipment Setup for optimized flow cytometry settings.

Note: Parasite multiplication rate of CM-treated cultures typically drops by 2-3 folds compared to parasites grown under non-inducing conditions. This is indicative for a successful induction of gametocyte production.

**Measurement of gametocytemia by flow cytometry**

18) Stain cells with SYBR Green by repeating step 16.

Note: At 62-70hpi, young gametocytes have acquired enough tdTom reporter for cytometry-based detection. As the intensity of the fluorescent signal continuously increases during early gametocyte development, detection at a later time point may be favorable. *CRITICAL Measurements should not be carried out after 86-94hpi to prevent detection of gametocytes formed during subsequent IDCs.

19) Perform flow cytometry to determine gametocytemia. Measure 400’000 events per well.

See Equipment Setup for optimized flow cytometry settings.

**Flow cytometry data analysis and hit selection**

Data analysis can be carried out using any cytometry analysis program (e.g. FlowJo).

20) To visualize flow cytometry data, export .fcs data files from the flow cytometer to the cytometry analysis program. Generate dot plots with Y2 intensity (tdTom fluorescence) on the Y-axis and B1 intensity (SYBR Green fluorescence) on the X-axis. Gate double negative (uRBC), SYBR Green positive (asexual parasite) and tdTom/SYBR double positive (gametocyte) cell
populations (see Figure 15b). Use the same gates for all samples from the same flow cytometry run.

21) Determine parasite proliferation rates for each well by dividing the proportion of SYBR Green positive cells (parasitemia) from step 17 by 0.3 (the starting parasitemia). Determine sexual conversion rates or, in case of testing compounds active against early gametocytes, gametocyte survival rates for each well by dividing the proportion of SYBR Green positive cells (parasitemia) from step 17 by the proportion of tdTom/SYBR double positive cells (gametocytemia) from step 19.

Note: Double negative (uRBC), SYBR Green positive (asexual parasite) and tdTom/SYBR double positive (gametocyte) cell populations separate clearly and can be gated without assistance of corresponding controls (see Figure 15b).

22) Calculate the mean rate of sexual conversion or gametocyte survival ± SD for all test conditions, as well as for the induced, non-induced and vehicle controls. *CRITICAL Confirm absence of significant effects of the vehicle on parasitemia, gametocytemia and conversion rate by comparing to induced and non-induced control conditions.

23) Assess the effect of probed perturbations on sexual conversion and/or gametocyte survival in relation to the induced control wells created in step 14 (defining maximal sexual conversion and 100% gametocyte survival, respectively) and non-induced control wells created in step 12 (defining baseline conversion).

24) Determine ‘hits’ according to self-determined thresholds.
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<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
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<tr>
<td>10</td>
<td>Not enough cells are obtained after synchronization to seed the required number of 96-well plates.</td>
<td>Parasitemia of the starting culture was too low, parasites proliferated poorly, or many cells were lost during synchronization.</td>
<td>Allow parasites to complete another IDC before returning to this step. Use fresh RBCs. It is possible to save the CM for at least 10 days at 4°C.</td>
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<td>17</td>
<td>Low parasite multiplication rate under non-induced conditions.</td>
<td>The RBCs may be too old or, for other reasons, do not support optimal parasite growth.</td>
<td>Repeat the assay with a fresh batch of RBCs. It may be beneficial to combine RBCs from multiple donors for culturing.</td>
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<td>17</td>
<td>Low parasite multiplication rate under CM-induced conditions.</td>
<td>Note that parasite multiplication rate of CM treated cultures typically drops by 2-3 folds compared to parasites grown under non-inducing conditions. This is indicative for a good induction of gametocyte production, rather than indicating a problem.</td>
<td>If the parasite multiplication rate drops below 2, it may be necessary to further optimize the assay by using different CM conditions (e.g., lower the concentration of the CM working solution from 90% to 87.5%).</td>
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<tr>
<td>19</td>
<td>Drug treatment induces a premature induction of the TdTom reporter at 24-32hpi.</td>
<td>Parasites show a faux induction response to drug treatment (see Anticipated results)</td>
<td>Perform gametocyte quantification at 86-94hpi. The transient signal from faux induced parasites is not detectable anymore at this time point (see Anticipated results).</td>
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<tr>
<td>19</td>
<td>CM-treatment fails to induce gametocytogenesis</td>
<td>Small changes in assay conditions may drastically alter the rate at which gametocytes are produced. While CM collected from cells at a parasitemia below 5.5% may lead to poor inductions, the application of other CM conditions may result in the complete killing of the experimental parasite population. Note that different strains may respond differently to CM-treatment.</td>
<td>We recommend performing an initial testing of the assay using CM working solutions at both 90% and 95%. This should allow achieving similar results to those shown in Figures 2. In case of low gametocyte production, increase concentration of the CM working solution (e.g. from 95 to 97.5%). Alternatively, increase the parasitemia of the culture used to produce the CM (see step 7). If CM treatment causes complete killing of the experimental cell population, lower the concentration of the CM.</td>
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Alternatively, lower the parasitemia of the culture used to produce the CM.

| 19 | Many gametocytes are produced under noninducing conditions. | Parasite culture conditions may have caused a self induction of gametocyte production. Cells of the parasite strain used may convert to the sexual pathway at a high intrinsic rate (high baseline conversion). | Repeat the assay by strictly maintaining the parasitemia of the experimental culture below 1% prior to seeding. Perform the assay with cell lines showing low baseline sexual conversion. |

Table 3. (Continued)
Timing

Day 1: Steps 1 to 3; first sorbitol synchronization (40 minutes)

Day 2: Step 4; second sorbitol synchronization (40 minutes)

Day 3: Steps 5 and 6; preparation of HP and LP cultures (30 minutes)

Day 5: Step 7; third sorbitol synchronization of LP culture (40 minutes).

Step 8; dilution and media exchange on HP culture (20 minutes).

Day 6: Step 9; collection of CM from HP culture (20 minutes)

Day 8: Step 10; fourth sorbitol synchronization of LP culture (40 minutes)

Steps 11 to 14; setting up the library screen (2 hours)

Day 9: Step 15; media exchange (30 minutes)

Day 10: Steps 16 and 17; flow cytometry measurement of parasitemia (~1 hour of preparation; cytometry requires ~1.5 hours per plate).

Day 11: Steps 18 and 19; flow cytometry measurement of gametocytemia (~1 hour of preparation; cytometry requires ~2.5 hours per plate)

4.5. Anticipated Results.

The presented protocol is designed to probe the effect of drugs and other changes in culture conditions on asexual parasite replication and early sexual development. Gametocytes are detected using the stage-specific expression of a fluorescent reporter. This renders the assay independent from gametocyte purification steps and enables screening the effects of perturbations on the earliest phases of gametocyte development including sexual commitment.
Tightly synchronized parasites of the Pf2004/164-tdTom cell line (others are available, see Materials) are induced for gametocytogenesis by applying a standardized CM working solution. As early as 62hpi, gametocytes expressing the red fluorescent reporter tdTom can be quantified by flow cytometry. Compared to non-induced cultures, CM-treated parasite populations are expected to increase sexual conversion rates by 40-80 folds to 14-24% (Figure 15b). Gametocyte development may be perturbed by introducing the compound of interest at any time during early maturation or even prior to sexual commitment. For example, dose response to drugs can be measured after administering the compounds at 24-32hpi (Figure 16). At this time point, notably, gametocytes cannot yet be physically separated from asexually replicating parasites. Also, because the assay does not require gametocytes to be purified, the effect of drug treatment on asexual parasites can be monitored simultaneously in the same well. Further, the low rate at which gametocytes are produced in Pf2004/164-tdTom cells under non-inducing conditions (see Figure 15a) allows identifying drugs and other perturbations that induce sexual conversion.

It is worth mentioning that some cytostatic drugs (including methylene blue and atovaquone) appear to deregulate stage-specific expression of gametocyte genes or induce reporter expression in cells that are not committed to the sexual pathway. This faux induction is transient, initiated at an earlier than expected time point, and only occurs at drug concentrations causing a near-complete block in asexual parasite replication (Figure 17). In case such faux induction is encountered (see step 17 of the protocol), we recommend deferring gametocyte quantification to 86-94hpi (see step 18). At this time point, faux induced cells have lost tdTom expression.
Figure 17. Drug screening: Dihydroartemisinin treatment affects asexually replicating parasites and early gametocytes. Shown is the effect of dihydroartemisinin (DHA) on asexual parasite multiplication (left panel) and early gametocyte development (right panel). Mitotic proliferation and gametocyte survival was monitored 48 hours after administering DHA at 24-32hpi. IC$_{50}$ values are 2.31nM (3D7/164-tdTom) and 1.77nM (Pf2004/164-tdTom) for asexual parasites and 0.99nM (3D7/164-tdTom) and 2.02nM (Pf2004/164-tdTom) for early gametocytes. Note that both cell lines reveal similar dose response curves. Curves were generated using a four-parameter non-linear regression fit.
Figure 18. Cytostatic drugs cause a faux activation of the tdTomato reporter. A. Bar graphs showing the proportion of red fluorescent parasites (Pf2004/164-tdTom) after treatment with different concentrations of dihydroartemisinin (upper panel) and methylene blue (lower panel). Except for the CM-induced control parasites, cells were cultured in absence of conditioned medium, i.e. under conditions that do not induce gametocyte production. High concentrations (compare with panel B) of methylene blue induce a short-lived activation of the tdTom reporter (highlighted in red). Note that this faux induction precedes proper reporter expression in early gametocytes of the control cells (highlighted in green). Drugs were added for 24 hours (from 24-32hpi of the preceding IDC to 0-8hpi). Bars show the mean of technical triplicates B. Effect of methylene blue on asexual parasite multiplication. Concentrations triggering a faux induction of the tdTom reporter are highlighted.
**A  Faux Induction**

Dihydroartemisinin

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**Methylene Blue**

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**B Dose response curve for Methylene Blue**

![Dose response curve](https://example.com/dose_response_graph.png)

**Figure 18. (Continued)**
ACKNOWLEDGEMENTS

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Author Contributions

I.G., N.M.B.B. and M.M. wrote the manuscript, with input from all coauthors. K.B. and M.M. conceived and developed the originally published protocol. I.G. conceived and performed preliminary experiments and N.M.B.B. conceived and performed later experiments leading to improvements in the protocol. N.M.B.B. and K.W. generated the data shown in the figures. All authors revised the manuscript and contributed to interpretation of the results.

Competing Financial Interests

The authors declare no financial interest.
Chapter 5. Conclusion
Sexual conversion is a critical step in the natural life cycle of *Plasmodium falciparum*. The timing and level of gametocyte production may have a great impact on the parasite’s ability to escape a human host, infect a mosquito and later disperse to additional hosts. Sexual conversion is also intertwined with parasite proliferation and virulence, both because asexual cycling and sexual development are mutually exclusive choices and because of shared regulatory mechanisms with parasite *var* genes\(^{20}\).

In the past five years, new discoveries have illuminated part of the sexual conversion pathway. Several of the involved processes may be targetable by drugs. We have learned that the defect in the gametocyte deficient strain F12, first reported in 1995\(^ {26}\), stems from its mutation in a gene coding for a transcription factor, and that this transcription factor (AP2-G) controls the last step of sexual conversion and the first step of “committed” sexual development in the schizont\(^ {18}\). The *P. falciparum* copy of heterochromatin protein 1, a widespread and conserved epigenetic regulator, was shown to repress AP2-G activation and thus sexual conversion in this species\(^ {21}\). HP1 was found to be recruited to loci targeted by an epigenetic code writer, HDA2, which also represses sexual conversion\(^ {20}\). Removal of the silencing on the AP2-G locus would be one of the key regulatory points in gametocyte production and is likely targeted by pro-conversion signaling.

Other recent discoveries point to potential participants in gametocyte conversion or very early development. The frequent observation that gametocyte production can be lost in long-term cultured strains was traced first to the deletion of a portion of Chromosome 9, then to deletion of the gene for GDV1, which is expressed in a subset of schizonts and localizes to the nucleus. This gene may function as a positive regulator of gametocyte conversion or
alternatively be required in very early sexual development. Researchers had observed that within a culture of clonal parasites exposed to the same environment some convert and some do not; this apparent stochastic aspect of the conversion decision can now be partially explained by AP2-G’s feedback loop, and perhaps partially explained by differential uptake of microvesicles. Signaling pathways are strongly suspected to be involved in connecting environmental signals to gametocyte formation. We have also learned that gametocytes sequester in bone marrow extravascular space during most of their development, and that the conversion decision may be linked to parasite migration to the bone marrow, though which of these occurs first is not yet clear.

The studies reported in this dissertation have added several new ideas to the knowledge in the field (Summarized in the model, Figure 19). A putative E3 ubiquitin ligase encoded by Pf13_0164 is probably a repressor of the pathway, likely acts upstream of AP2-G to prevent its transcription in asexually committing cells, and may regulate levels of HP1 protein in the nucleus. Parasite-conditioned medium and microvesicles released from infected erythrocytes can induce other cells to convert and may carry parasite density information. Chapter 4 provides the research community with an effective protocol for measuring parasite growth and gametocyte production in response to both stimulatory and inhibitory agents. The PB-60 mutant presented in Chapter 2 also highlights the common theme that some of the genes repressing sexual conversion are also required for normal cell cycle progression, suggesting potential links between conversion and cell cycle regulation. The disrupted genes in three additional piggyBAC hit strains presented in Chapter 2 may also be involved in gametocyte
Figure 19. Model of the potential place of Pf13_0164 and of microvesicles in the sexual conversion pathway. As a likely E3 ubiquitin ligase, Pf13_0164 could transfer one or more ubiquitin units to HP1 and regulate its activity, interactions or localization, potentially by causing it to move from the cytoplasm to the nucleus (Chapter 2). These hypothesized interactions are depicted with dotted lines. Alternatively it could ubiquitinate another protein that acts upstream of transcription factor AP2-G to suppress gametocyte conversion. In the nucleus, HP1 binds to H3K9me3 marks and silences AP2-G, blocking conversion. Positive regulators of the conversion pathway are predicted to include components of classic signaling pathways and to integrate internal stress and environmental signals including parasite-derived microvesicles transferred among cells (Chapter 3). These pro-sexual conversion signals would counteract or lead to the removal of the epigenetic repression machinery and allow transcription of AP2-G and early gametocyte genes. AP2-G, Pf14_0744 and Pf14_0748 are depicted in a silenced state.
Environmental stress signals

Internal stress signals

Pro Conversion Signaling

Pf13_0164

ubiquitin

HP1

H3

AP2-G

erythrocyte

parasite cytoplasm

parasite nucleus

Figure 19. (Continued)
conversion; the disrupted gene *Pff0295c* (mutant PB-65) is especially of interest as a possible repressor of conversion.

The known and suspected components of the conversion pathway include multiple potential drug targets. E3 ubiquitin ligases are the targets of two approved anticancer drugs and >10 drugs in clinical or preclinical development; because of their substrate specificity it is often possible to target an individual or a small number of E3 ligases (reviewed Skaar et al. 2014\(^{112}\)). Epigenetic regulatory proteins, including histone code writers, erasers and readers, are of great interest as drug targets: two histone deacetylase inhibitors are approved and others are in clinical development for cancer or inflammation; drugs that inhibit bromodomain containing proteins, histone acetylases, histone methyltransferases, and lysine demethylases are the subject of laboratory research or preclinical development. In light of the importance of HP1, HDA2 and AP2-G, additional epigenetic proteins will likely turn out to impact conversion. Microvesicle release and uptake are both thought to rely on parasite-derived mechanisms and could be potentially targeted by drugs once the details of these processes are more clear.

Recent discoveries related to sexual conversion raise many questions, including the adaptive role of this life cycle transition in parasite strategy. Why is sexual conversion an inducible phenotype instead of pre-programmed as are the other *P. falciparum* life cycle transitions? Why might it be subject to variation and regulation by the environment instead of always occurring at a fixed rate within a population? Increasing sexual conversion rates in *P. falciparum* clearly serves to provide transmission stages for mosquito uptake, but it could also serve as a way to restrain asexual expansion when high parasitemia is dangerous to the parasite population or the host by redirecting parasites to a nonreplicating developmental stage. Its
regulatory mechanisms could thus respond to signals indicating adverse environments or dangerously high parasitemia. Other possibilities include a parasite strategy of switching to a more durable, longer-lived form when conditions indicate asexual parasites would be unlikely to survive; and co-evolution with epigenetic mechanisms controlling antigenic variation which could serve to alternately shield asexual parasites and gametocytes from immune responses. Meanwhile, maintenance of low conversion rates under non-inducing conditions could allow parasites to outcompete other clones in an infection via rapid blood-stage cycling, or could increase the asexual population with the goal of increasing the numbers of gametocytes produced for transmission later in the infection. Thus, flexible and environmentally responsive cell fate switching could be key for parasite survival and success in the wild.

With so many discoveries in recent years, it may be possible soon to make sense of the entire gametocyte conversion pathway at a molecular level. Researchers may be able to identify the interaction partners and cellular targets of the known and hypothesized participants in the conversion pathway in order to link the pathway together from start to finish. Major questions remain, including how best to use these findings to prevent transmission and the possibilities for modifying the conversion pathway with drugs. In vitro and molecular findings will need to be linked to findings from real human infections and to the behavior of parasites within their microenvironments in their hosts. A full understanding of this critical life cycle decision can lead to the development of strategies and interventions for reducing malaria transmission and disease burden.
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**Malaria-Infected Erythrocyte-Derived Microvesicles Mediate Cellular Communication within the Parasite Population and with the Host Immune System**

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**SUMMARY**

Humans and mice infected with different *Plasmodium* strains are known to produce microvesicles derived from the infected red blood cells (RBCs), denoted RMVs. Studies in mice have shown that RMVs are elevated during infection and have proinflammatory activity. Here we present a detailed characterization of RMV composition and function in the human malaria parasite *Plasmodium falciparum*. Proteomics profiling revealed the enrichment of multiple host and parasite proteins, in particular of parasite antigens associated with host cell membranes and proteins involved in parasite invasion into RBCs. RMVs are quantitatively released during the asexual parasite cycle prior to parasite egress. RMVs demonstrate potent immunomodulatory properties on human primary macrophages and neutrophils. Additionally, RMVs are internalized by infected red blood cells and stimulate production of transmission stage parasites in a dose-dependent manner. Thus, RMVs mediate cellular communication within the parasite population and with the host innate immune system.

**INTRODUCTION**

*Plasmodium falciparum* causes more than 200 million cases of malaria and more than 1 million deaths each year (Snow et al., 2005). Rapid asexual amplification of parasites in human red blood cells (RBCs) can result in severe and life-threatening disease, while development of sexual stages or gametocytes is required for successful parasite transmission to the mosquito vector. Here we show that microvesicles are quantitatively released by parasite-infected RBCs and transferred between parasites, regulating the production of malaria transmission stages.

Microvesicles (MVs) are small vesicles (0.1–1 μm in size) that are produced by direct plasma membrane blebbing. MVs can contain proteins, RNA, and even organelles and act as messengers between cells (Skog et al., 2008). In mammalian cells, the rate of MV release is usually low but can be increased by cell activation or apoptosis. Increased MV production by human cells has been observed in a variety of conditions, including cardiovascular disease, arthritis, and thalassemia, and tumor cells can constitutively shed a large number of MVs (Cocucci et al., 2009).

In recent studies, malaria patients infected with either *P. falciparum* or the related human parasite *P. vivax* showed elevated levels of MVs derived from platelets and RBCs (Cocucci et al., 2010; Nantakomol et al., 2011). MV numbers were increased in patients suffering from severe disease and correlated with peripheral blood parasitemia. After antimalarial treatment, the level of MVs decreased rapidly and continued to decrease further between days 3 and 14 (Nantakomol et al., 2011). Flow assays using antibodies against the parasite antigen RESA, which is localized underneath the infected RBC (iRBC) membrane, have suggested that this protein is present in MVs from malaria patients (Nantakomol et al., 2011). Studies in the rodent malaria model (*P. berghei*) have provided evidence that MVs derived from iRBCs (RMVs) induce host inflammatory responses and contribute to pathology during malaria infection. These studies have shown that RMVs are elevated during infection and that they have a potent, Toll-like receptor-mediated proinflammatory effect on macrophages (Couper et al., 2010). Here we demonstrate that RMVs are quantitatively released from iRBCs during development of the human malaria parasite *P. falciparum*. The majority of RMVs are released very late in the asexual cycle, and they contain both human- and parasite-derived proteins. Additionally, RMVs are internalized by infected RBCs and stimulate production of transmission stage parasites in a dose-dependent manner.
P. falciparum-derived proteins and other cargo. We provide evidence that RMVs are both immunostimulatory and act as messengers between iRBCs. RMVs are transferred among iRBCs and alter the production of transmission stages within a population. Our studies provide a rationale for systematic investigation of the role of RMVs in malaria pathogenesis and as a mediator of cell-cell communication during the parasite life cycle.

RESULTS

RMVs Are Released from RBC In Vitro Cultures Infected with P. falciparum

To characterize the biogenesis, composition, and cellular targets of RMVs in the human malaria parasite P. falciparum, we used an in vitro model in human RBCs. Imaging flow cytometry analysis revealed that a large number of particles present in cell suspension are smaller than RBCs. Microscopic inspection of individual objects clearly supported cytometric classification into three distinct populations based on size differences: clusters of RBCs ("rosettes," gate M in Figure 1A), single red blood cells and ghosts (S, probably also containing debris), and small particles that looked like vesicles in the corresponding bright field images (RMV, shown in yellow in Figure 1A).

We developed a protocol for the purification of RMVs from culture supernatant (i.e., what is also referred to as parasite conditioned medium) based on differential centrifugation, filtration, and a 60% sucrose cushion (Figure S1A available online). The protocol was optimized by analysis of samples from individual purification steps with imaging flow cytometry analysis, microscopy, and western blot. Digestive vacuoles and merozoites were collected in the 3,600 g pellet as demonstrated by Giemsa staining and western blot. The 10,000 g pellet mostly contains membrane debris as suggested by the presence of spectrin and the absence of hemoglobin. The final RMV pellet is enriched in stomatin and hemoglobin (Figure S1B), and imaging flow cytometry analysis of this fraction demonstrated that the purification procedure resulted in enrichment of vesicles to >95% of all detected events (Figure 1A). To further confirm the vesicular nature of these objects, we stained them with calcein-AM and annexin V. (Figure 1B). Calcein-AM is a membrane-permeable MV marker that becomes fluorescent and trapped in the cytosol upon cleavage by esterases. RMV labeling with calcein-AM dye demonstrated that approximately 80% of events in the final fraction are positive, suggesting presence of esterase activity within RMVs. Colabeling with annexin V confirmed the vesicular nature by binding to phosphatidyl serine on the RMV surface.

The final fraction of purified RMVs was analyzed by transmission electron microscopy, demonstrating vesicular shape and size in the range between 100 and 400 nm (Figure 1C). We directly observed the release of RMVs from infected red blood cells during time-lapse imaging experiments in which live parasite cultures were imaged every 2 min over 2 hr (Figure 1D and Movie S1). Imaging revealed that multiple vesicles at different stages of formation exist simultaneously in single iRBCs, suggesting significant RMV production during at least some parts of the parasite cycle.

RMVs from Infected RBCs Contain Parasite Material

To characterize the properties and function of RMVs, we first purified vesicles from four P. falciparum strains and investigated their protein content by separation of samples on an SDS-PAGE gel followed by Coomassie staining (Figure 2A). We observed a similar protein pattern across RMV fractions derived from all parasite strains, which differed from those of uninfected control samples and from isolated parasite schizont stages (Figure 2A).

To detect potential parasite proteins on RMVs, we tested pools of immune sera from malaria patients for reactivity with the same set of samples (Figure 2A). The sera were previously collected from adults in two highly endemic areas in Uganda and Tanzania, as part of the Millennium Village project. Both serum pools strongly reacted with multiple proteins in the infected RMV samples from all parasite strains analyzed, but not with any preparation from uninfected RBCs (uRBCs; Figure 2B). The pattern of reactive bands in RMVs was also different from those present in the schizont preparation. Together, these data suggest that RMVs have a distinct composition and that those derived from iRBCs additionally contain a specific set of parasite antigens.

Proteomic Profiling Reveals that RMVs Are Enriched in Membrane-Associated Parasite Antigens

To identify the parasite and host proteins present in RMVs, we characterized purified RMV samples using mass-spectrometry-based proteomic profiling. We analyzed RMVs derived from two culture-adapted parasite strains (3D7 and CS2) and from uRBCs as a control. In all the three preparations, we found that the most abundant RBC proteins were components of RBC lipid rafts such as stomatin and band 3, as well as several Membrane-Associated Parasite Antigens.

Figure 1. Initial Characterization of RMVs from P. falciparum-Infected Red Blood Cells

(A) Analysis of events by ImageStream before and after RMV purification. Nonfractionated cell suspension from in vitro culture was analyzed and three populations (S, M, and RMV) were differentiated on the basis of intensity and area. Image analysis demonstrates that M consists of clusters of multiple RBCs ("rosettes"), S consists of single RBCs, and RMV consists of smaller events of vesicular nature. The RMV purification protocol resulted in an enrichment to >95% of all events in the RMV gate.

(B) Calcein and annexin V labeling of RMVs. ImageStream analysis of calcein-AM and annexin V antibody staining demonstrates double labeling of RMVs (left panel). By flow cytometry, approximately 55% of all events are double positive, although the real number is likely higher due to the limited sensitivity and size cutoff of flow cytometry (right panel).

(C) Characterization of the RMV population by electron microscopy. Analysis of fixed RMVs from P. falciparum in vitro cultures reveals vesicular structures of 100-400 nm.

(D) Live imaging of RMV release. Release was captured by time-lapse microscopy of infected RBCs labeled with the surface marker CellVue, using 2 min intervals over the course of 2 hr (top panel). Serial images were taken across the z plane by fluorescence microscopy of individual cells (bottom panel). In both cases multiple RMVs can be observed emerging from a single infected RBC.

See also Figure S1 and Movie S1.
carbonic anhydrases (Figure 3A and Table S1), which are known to be enriched in MVs derived from RBCs (Rubin et al., 2008). To determine whether RMVs are enriched in particular classes of proteins, we stratified the hits from the proteomic analysis by Gene Ontology (GO) localization term enrichment analysis. This analysis revealed that extracellular- and vesicle-associated moieties are the most enriched in RMVs. By both total absolute protein content and GO localization, there was no apparent difference in RBC protein content between infected and uninfected RMVs (Figures 3A and S2).

We identified more than 30 parasite proteins in the RMV preparations from 3D7 and CS2 parasite strains (Figure 3B and Table S1). These proteins mainly belong to two classes: proteins associated with RBC membranes and proteins involved in parasite invasion into RBCs (Figure 3C). The first class is represented by components of the Maurer’s clefts (SBP1, Rex1/2, MAHRP1/2, and PfMC-2TM), proteins linked to the RBC surface membrane (Clag3.1/2, RESA, and MESA), and proteins associated with the parasitophorous vacuole membrane (PVM; Exp-1 and Etramp2). The second class is represented by erythrocyte binding antigens (EBA-175 and EBA-181, which bind to glycophorins during merozoite invasion before being shed) and moity proteins (RhopH2/H3 and Rap2).

To further corroborate the enrichment of some of the proteins found in RMVs, we performed immunoblots of RMV samples using specific antibodies (Figure 3D). These experiments confirmed enrichment of stomatin and partial depletion of spectrin, as well as presence of the secreted parasite proteins RESA, SBP1, and, at relatively lower abundance, the PVM marker Exp-1. Importantly, we did not identify any markers for components of the parasite-induced knob complex on the IRBC surface, including KAHRP and PiEMP1, or resident parasite proteins such as the ER marker Bip. Together with the presence of RBC lipid raft proteins on RMVs, this finding implies that RMVs arise by blebbing from specific subdomains within the RBC membrane. We also consistently detected the same parasite markers across the two genetically diverse reference parasite strains, 3D7 and CS2, suggesting that the composition of RMVs is conserved in P. falciparum.

To determine the distribution and orientation of host and parasite proteins within RMVs, we performed enzyme protection assays with purified RMVs. RMVs were treated with the detergent Triton X-100 (TX-100) and with either trypsin or proteinase K. These experiments demonstrated that glycophorin C is present on the RMV surface, as can be expected if RMVs bleb off the RBC surface. We found that stomatin and two parasite proteins, Exp-1 and SBP1, were protected (Figure 3E). It has been previously shown that the C-terminal tail of the Maurer’s cleft protein SBP1 faces the RBC cytoplasm, whereas the N terminus is located in the lumen (Blisnick et al., 2000). The absence of any processed product in our protection assays therefore suggests that the Maurer’s cleft membrane is present within the RMV, and it independently confirms RMV integrity in the preparation. We also observed that EBA-175 and EBA-181, two of the most abundant parasite proteins identified by RMV proteomics, are efficiently digested by both trypsin and proteinase K even in the absence of TX-100. This demonstrates that the proteins are only peripherally associated with RMVs upon shedding. The large immunogenic EBA ectodomains face the inside of the microneme and would therefore be protected from enzyme digestion in the case of microneme contamination in our preparation. In conclusion, proteomic profiling demonstrates that RMVs from IRBCs contain (1) a set of enriched RBC proteins and (2) parasite antigens derived from the RBC surface and internalized membranes, in particular from the Maurer’s clefts.

**Size and Density Differentiate RMVs from Other RBC-Derived Material**

In other systems, microvesicles have been defined as vesicular particles with a specific density in sucrose gradients and a size...
range from 0.1 to 1 μm (Muradidharan-Chari et al., 2010). To determine whether our preparations from iRBCs and RBCs represent homogenous populations of microvesicles, we prepared culture supernatants by ultracentrifugation and layered pellets on a continuous linear sucrose gradient. We obtained ten fractions and analyzed them for protein content by BCA
and western blot analysis with the subset of parasite and host markers described above. We demonstrated that both protein content and specific RMV markers peaked in fractions 3 and 4 (Figures 4A and 4B). These fractions represent a density of 1.221–1.198 g/cm$^3$, which is within the range of densities for MV preparations from other cell types and is higher than exosome density (1.08–1.22 g/cm$^3$) (Raposo et al., 1996). Probing with the merozoite marker AMA-1 was negative, again confirming absence of merozoites and homogeneity of the vesicular population in the preparations. Immunoblotting demonstrated the presence of the putative resident RMV proteins EXP-1, RESA, and SPB-1, as well as stomatin and band 3 in fractions 3 to 5. To independently confirm particle abundance across the sucrose fractions and to determine whether RMVs were also homogenous in size, we analyzed RMVs using a nanoparticle tracking technology (NanoSight), which quantifies particles between 0.1 and 1 μm. This analysis confirmed that RMVs peak in sucrose fractions 3 and 4 in preparations from both RBCs and iRBCs, with the majority of RMVs between 100–150 nm in size. The iRBC preparation showed a longer tail in the size distribution, suggesting existence of an additional subset of RMVs with slightly larger size, between 150 and 250 nm, but with the same density. A similar size distribution was obtained by flow cytometry with size beads and calcein-AM staining (data not shown).

**RMV Release Peaks during Schizogony but before Parasite Egress**

To determine whether vesicles are constitutively released or whether their release is linked to a particular phase in the parasite cycle, we performed a series of kinetic experiments. We collected supernatants from highly synchronized parasite cultures every 12 hr starting at the ring stage and isolated vesicles for total particle quantification by size (using NanoSight), protein content (using BCA) and protein composition (using western blot analysis). These analyses suggested that RMV release increases steadily during the parasite cycle and peaks late during schizogony or shortly thereafter. This dynamic of release coincided with the emergence of a prominent vesicular subpopulation of 150–250 nm in the iRBC preparation only. Immunoblotting
revealed concomitant peak levels of parasite RMV markers such as RESA and SBP1 and the host markers spectrin and band 3 in the vesicular fraction from late schizogony (Figure 5A).

To further distinguish between vesicle release during parasite development or during egress, which can include release of parasite-derived organelles, we used two complementary approaches to either genetically or chemically inhibit parasite egress. We performed a conditional knockdown of the P. falciparum calcium-dependent protein kinase 5 (PfCDPK5) results in a block of parasite egress (Divoin et al., 2010). Using this conditional knockdown line, we collected supernatants and analyzed vesicle production over time in the presence or absence of PfCDPK5 protein. Our results strongly suggest that RMVs are released before and not during parasite egress (Figure 5B). Importantly, similar experiments with the cysteine protease inhibitor e64, an inhibitor of parasite egress (Millholland et al., 2011), could phenocopy this effect (Figure 5B). Altogether, these data demonstrate that the peak release of RMVs from iRBCs occurs shortly before egress (i.e., within the last 6–8 hr of the parasite asexual cycle). RMVs are therefore distinct from recently described postnuclear vesicles that are released upon egress of parasites from the red blood cell (Millholland et al., 2011). This conclusion is supported by the absence of markers for these postnuclear vesicles such as the parasite antigens Sera-5 and Sera-6 in RMVs by proteomics (Table S1).

To quantify RMV release from iRBCs and RBCs, we designed an experiment using two lipophilic membrane dyes, which are readily incorporated into RBCs: PKH26, which emits red light, and PKH67, which emits green light. This combination of dyes allows tracking of distinct particle or cell populations. We labeled highly synchronized trophozoite stage iRBCs with PKH67 and mixed them with uRBCs that we labeled with PKH26. Using flow cytometry and fluorescence microscopy, we monitored release of red and green RMVs during the remainder of the parasite cycle and after reinvasion, in the presence or absence of e64, (see Figure 5C, panel I, for the experimental setup). Using different mixtures of labeled iRBCs and RBCs, we determined the relative contribution of iRBC-derived RMVs to total RMV production. This demonstrated that iRBCs release about ten times more RMVs than uRBCs, and e64 experiments independently confirmed that these RMVs are released before egress (Figures 5C and S3).

**RMVs Derived from iRBCs Activate Host Monocytes and Neutrophils**

So far we have provided a thorough examination of the composition, biophysical properties, and release kinetics of RMVs. In the next series of experiments, we aimed to examine their potential physiological role(s). Recent studies in the rodent malaria model have demonstrated that RMVs derived from P. berghei iRBCs strongly activate the innate immune response through macrophage stimulation and cytokine production, suggesting an immunomodulatory role in malaria pathology (Couper et al., 2010). To investigate the potential of Plasmodium falciparum RMVs to modulate the innate immune response in human malaria, we analyzed their effect on human peripheral blood mononuclear cells (PBMCs), macrophages, and neutrophils derived from healthy, malaria-naïve donors. PBMCs were incubated with RMVs from uninfected and infected RBCs to determine which cell type was activated and whether pro- or anti-inflammatory cytokines were induced. We measured the cell markers CD3 (T cells), CD19 (B cells), and CD14 (monocytes) in combination with the activation markers CD54, CD25, CD40, CD163, CD86, and CD36 by flow cytometry; cell viability was assessed by annexin V and propidium iodide staining. These experiments demonstrated that monocytes (CD14+) are the main targets of RMVs (Figures 6A and S4A–S4C). Specifically, they showed peak levels of the activation markers CD40, CD54, and CD86 and downregulation of CD163 upon stimulation with RMVs from iRBCs but not from uRBCs.

For assessment of the effect of RMVs on human macrophages, monocytes were isolated from PBMCs, differentiated into macrophages, and activated with LPS. Quantitative RT-PCR (qRT-PCR) analysis demonstrated that RMVs from iRBCs can activate the proinflammatory cytokines interleukin-6 (IL-6), IL-12, and IL-1 and the anti-inflammatory cytokine IL-10 in a dose-dependent manner (Figure S4D). We confirmed these results by demonstrating that the ex vivo stimulation of the activation markers CD40, CD54, and CD86 (CD14+) are the main targets of RMVs (Figures 6A and S4C). Importantly, macrophage activation depends on the active uptake of RMVs, as demonstrated by microscopy of RMV uptake (Figure 6C), and cytokine induction was inhibited in presence of cytochalasin D, an inhibitor of phagocytosis (Figure 6D).

To probe the role of IRBC-derived RMVs on human neutrophils, we incubated freshly isolated human neutrophils with purified RMVs inside a microfluidic device with small migration channels (Butler et al., 2010). Brief exposure (30 min) to RMVs from iRBCs but not from uRBCs activated neutrophils to spontaneously move, even in the absence of any guiding chemotactic gradients (Figure 6E). We also found that neutrophils preincubated with RMVs from uRBCs migrated at a slower rate compared those preincubated with RMVs from iRBCs or untreated controls. Together, these data demonstrate that RMVs from iRBCs but not from uRBCs can strongly stimulate cells of the innate immune system.

**RMVs Are Internalized into iRBCs and Induce Transmission Stage Formation**

In the PKH experiments described in Figure 5C, we noted that in some cases fluorescence from PKH67-labeled vesicles could also be found inside iRBCs, suggesting that they had been incorporated into the cell. To further investigate this observation, we performed a series of RMV uptake experiments and phenotypic assays. First, we investigated uptake of labeled RMVs by microscopy, immunoelectron microscopy, and flow cytometry. Live fluorescence microscopy revealed that PKH67-labeled RMVs are efficiently incorporated into iRBCs and eventually accumulate in the parasite and at its nuclear periphery (Figure 7A, panel I). Uptake appears to be specific to iRBCs, since RMVs were found mostly bound to the surface of uRBCs (Figure 7A, panel II). Quantification of uptake with PKH67-labeled RMVs also demonstrated that those derived from iRBCs are incorporated at significantly higher rates than those from uRBCs (Figure 7A, panel III). Notably, only a subset of iRBCs internalizes RMVs even at very high concentrations, suggesting that not all iRBCs are equally receptive for uptake. To visualize RMV uptake on
an ultrastructural level, we performed immune electron microscopy of RBCs after incubation with biotinylated RMVs from infected RBCs. In concordance with live microscopy, we observed the presence of labeled vesicles in the host cell cytoplasm and in the parasite (Figure 7B). These internalized RMVs are surrounded by additional membranes, suggesting that phagocytosis-like mechanisms are operational in infected RBCs.

To determine whether RMV uptake has a phenotypic effect on parasite growth, we treated ring-stage parasites with a serial dilution of purified RMVs and investigated parasitemia after one replication cycle (Figure 7C). While we did not observe a significant alteration in growth rates after 48 hr, we noted the emergence of increased numbers of gametocytes in the parasite culture at later time points. Sexual stages, or gametocytes, are formed from asexual parents at low rates of <0.1%–15% per re-invasion round in vitro. In vivo gametocyte production appears to cover an equally large range (Alano, 2007). Upon maturation, gametocytes are transmitted to a mosquito vector during a blood meal, where they undergo fertilization and further development.

It has been reported that parasite-conditioned medium (i.e., the parasite culture supernatants that we use for RMV isolation) can increase the proportion of gametocytes formed (Dyer and Day, 2003), and such conditioned medium is commonly used as a stimulus to increase gametocyte production under in vitro conditions (Fivelman et al., 2007). The factor(s) responsible for this effect have not yet been identified. To test whether RMVs from conditioned medium have a gametocyte-inducing effect, we quantified gametocyte production in 3D7 parasites upon addition of conditioned medium or purified RMVs derived from the same conditioned medium. These experiments revealed that RMVs derived from iRBCs stimulated increased gametocyte production in a titrable fashion and similar to conditioned medium from late stage parasite cultures, whereas RMVs from uRBCs had a less pronounced effect and fresh medium produced minimal levels of gametocytes (Figure 7D). Together, these experiments strongly suggest that RMVs from iRBCs are the active component in conditioned medium that stimulates gametocyte production and that the concentration of these RMVs can regulate gametocyte production on a population level. These observations are in line with the findings presented by Regev-Rudzki et al. (2013).

DISCUSSION

Exosomes and MVs are involved in many physiological processes, and numerous pathological conditions increase their rate of formation. Previous studies have demonstrated that MVs originating from endothelial cells, platelets, and RBCs are present during malaria infection and have suggested a link to the host immune response to malaria. In the present study, we show that P. falciparum-infected RBCs release MVs in a quantitative fashion in the environment. These RMVs contain parasite antigens and are highly immunogenic, stimulating human PBMCs and primary macrophages and activating neutrophil migration. Finally, we provide evidence that RMVs can be internalized by iRBCs and modulate formation of parasite transmission stages. Our studies suggest that RMVs play major roles in the biology of Plasmodium parasites and in the pathogenesis of human malaria.

Establishment of a working protocol for RMV isolation from parasite culture supernatant (i.e., conditioned medium) provided us with the opportunity to define RMVs by size, density, and composition. Interestingly, within the small fraction of parasite proteins identified in RMVs, proteins resident in the Maurer’s clefts are enriched. Maurer’s clefts are parasite-induced membrane structures in the host cell that serve as platforms for surface deposition of parasite antigens. We also identified several surface antigens in RMVs; however, RMVs lack components of the knob, a P. falciparum cytoadherence complex on the iRBC surface, such as PIEMP1 and KAHRP. This suggests that RMVs are formed from Maurer’s cleft structures and from iRBC subregions that exclude knobs. We have also performed a series of complementary experiments to unambiguously demonstrate that RMV release occurs before egress and coincides in timing with the observed disappearance of Maurer’s clefts (Grüning et al., 2011). Interestingly, parasites employ host actin to generate a cytoskeleton within the cytoplasm of the iRBC that connects the Maurer’s clefts with the host cell.
Figure 6. Immune Stimulatory Activity of RMVs

(A) Activation of PBMCs. Quantification of activation markers per cell type by flow cytometry. Multiple markers are upregulated in monocytes as well as CD86 in B cells.

(B) Activation of macrophages. TNF-α and IL-10 production by stimulated macrophages was measured by performing ELISA on the supernatant. Data are presented as mean ± SD and are representative of three independent experiments.

(C) RMV uptake into human macrophages. Human macrophages were stimulated for 1 or 2 hr with RMV isolated from IRBC culture supernatants in the presence or absence of cytochalasin D (2.5 μM); cell membranes were stained with fluorescent Cell Mask Deep Red (red), and nuclei were stained with Hoechst 33342 dye (blue), followed by analysis of PKH67-labeled RMV uptake (green). Scale bars represent 10 μm. Multiple internalized green vesicles can be observed in the absence of CytD, while RMVs are only present on the macrophage surface upon CytD treatment (white arrows).

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Our experiments suggest that production of RMVs from iRBCs is approximately 10-fold higher than from uRBCs within the time frame of the asexual parasite cycle. This confirms data from a recent study suggesting that iRBCs produce roughly 13 times more RMVs than uRBCs (Wang et al., 2012). We demonstrate that macrophages can phagocytose RMVs from iRBCs after opsonization with serum and that these RMVs strongly induce cytokine release in PBMCs and macrophages. Excessive or deregulated inflammatory responses to malaria, including high levels of TNF-α or inadequate production of regulatory (anti-inflammatory) cytokines such as IL-10 and TGF-β, are associated with the development of cerebral malaria in both human infections and rodent models of disease. Several highly immunogenic parasite factors such as free GPI, hemozoin, and DNA are released from parasites and activate the innate host response; however, the exact mechanism of their secretion is not known. It remains to be shown whether these factors are indeed present in RMVs and contribute to the observed activation of the host immune system. A local proinflammatory response might be beneficiary for the parasite since it allows the cells to sense population density and coordinate activities including production of the secondary metabolite pyocyanin (Mashburn and Whiteley, 2005). Autoregulation of proliferation may be achieved by density-dependent differentiation into nondividing stages. In the etiological agent of sleeping sickness, Trypanosoma brucei, an as yet unidentified stumpy-inducing factor (SIF) mediates density sensing and regulates the formation of nondividing stumpy forms of the parasite, which are required for transmission to the tsetse fly vector (Reuner et al., 1997).

In some systems, a key mechanism by which MVs mediate cellular communication is through microRNA (miRNA). For example, exchange of miRNAs between antigen-presenting cells and T cells occurs at the site of immune synapses and regulates gene expression in the recipient cells (Mittebrunn et al., 2011). Endothelial cells secreting miRNA-containing MVs regulate atherosclerosis formation in the aorta by targeting smooth muscle cells (Hergenreider et al., 2012). Although the underlying mechanism by which RMVs influence MVs regulatory mechanisms remains to be determined, the presence of additional membranes surrounding RMVs after transfer into the parasite suggests that intact vesicles are internalized through phagocytosis-like mechanisms. This process may be related to uptake of RBC material through the parasite cytostome, which can deliver vesicles to the parasite food vacuole (Abu Bakar et al., 2010), or to autophagy-related mechanisms. We show that RMVs concentrate around the parasite nucleus. It has recently been demonstrated that parasite-infected sickle cell RBCs produce miRNAs that can translocate into the parasite where they interfere with protein translation and induce formation of gametocytes (LaMonte et al., 2012). We have identified small RNAs including miRNA in RMVs from iRBCs (Figure S6), and it is therefore conceivable that specific miRNA species are also involved in the gametocyte formation phenotype we observed in the internalization experiments. Altogether our study reveals that MVs derived from P. falciparum-infected red blood cells possess important functions in immunomodulation and cellular communication. These findings provide a foundation for further investigations into the molecular targets of RMVs and their contents after uptake into
Figure 7. RMV Uptake and Gametocyte Production
(A) Live analysis of RMV internalization into RBCs. PKH67-labeled RMVs are incubated with RBCs, and uptake into live cells is analyzed after 1 and 2 hr incubation by fluorescence microscopy (I and II) or flow cytometry (III). Uptake into infected RBCs can readily be detected as vesicular structures accumulating in the host cell and at the parasite periphery after 1 hr of incubation, and in a perinuclear location after 2 hr (white arrows). Flow cytometry data are presented as mean ± SD of four independent experiments.

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infected cells and the implications of this cell-cell communica-
tion for the pathology of human malaria.

EXPERIMENTAL PROCEDURES

Experimental procedures are described in further detail in the
Supplemental Experimental Procedures.

Preparation of RMVs and Other Cellular Samples

RMVs were isolated by a combination of sequential rounds of centrifugation at
increasing speed, high-molecular-weight cutoff filters, and a one-step sucrose
gradient. Purification of resulting samples was controlled by imaging flow cytometry
and western blot analysis of different fractions. Final fractions were further
characterized by electron microscopy and 2D gel electrophoresis. Detailed
description of purification protocol including cellular controls, as well as
analysis by imaging flow cytometry, flow cytometry, and western blot is
included in the Supplemental Experimental Procedures.

Proteomic Profiling of RMVs

Proteomic profiling analyses were performed similarly to what we described
previously (Dicker et al., 2010) and as detailed in the Supplemental
Experimental Procedures. Isolated MVs were either fractionated by one-
dimensional PAGE with the resulted fractions then being in-gel digested (first repli-
cate) or were in-solution digested without prefractionation (second repli-
cate) prior to nano LC-MS/MS-based proteomic profiling performed in tech-
nical duplicates for each digest, followed by comparative analysis of identified proteins and gene ontology terms corresponding to detected proteins. Detailed proteomics procedures and protocols are described in the Supple-
mental Experimental Procedures.

Dynamics of RMV Release

Quantitative and temporal dynamics of RMV release across the intraerythro-
cytic P. falciparum cycle were captured in time course experiments by collec-
tion of samples at specific intervals. Chemical and genetic perturbations were
determined to whether RMV release peaks before or during parasite
egression. Sample analysis was performed by a combination of western blot
and nanoparticle tracking technology (NanoSight). To determine the relative
amounts of RMVs released from iRBCs versus uRBCs from within a parasite
culture, we also labeled cells with the two lipid dyes PKH26 and PKH67, using
flow cytometry as readout. Detailed description of these experiments is avail-
able in the Supplemental Experimental Procedures.

Macrophage and Neutrophil Activation Assays

Macrophage stimulation was assayed with series of activation markers and
cytokines, by a combination of qRT-PCR, flow cytometry, and ELISA assays. Neutrophil activation was measured microscopically in migration assays
within microfluidics devices. Detailed procedures are included in the Supple-
mental Experimental Procedures.

RMV Internalization into IRBCs and Phenotypic Analysis

Identification in the presence or absence of inhibitory compounds was done
with labeled RMVs and assayed by fluorescence and immunoelectron micro-
copy as well as flow cytometry. Phenotypic analysis upon RMV incubation
was performed in 96-well format with standard assays for parasite growth
and gametocyte production (Buchholz et al., 2011). Further details are included in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental
Procedures, one movie, and one table and can be found with this article online
at http://dx.doi.org/10.1016/j.chom.2013.04.009.

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