CRISPR/CAS9 genetic modification of plasmodium falciparum and transgenic parasites in malaria vaccine research

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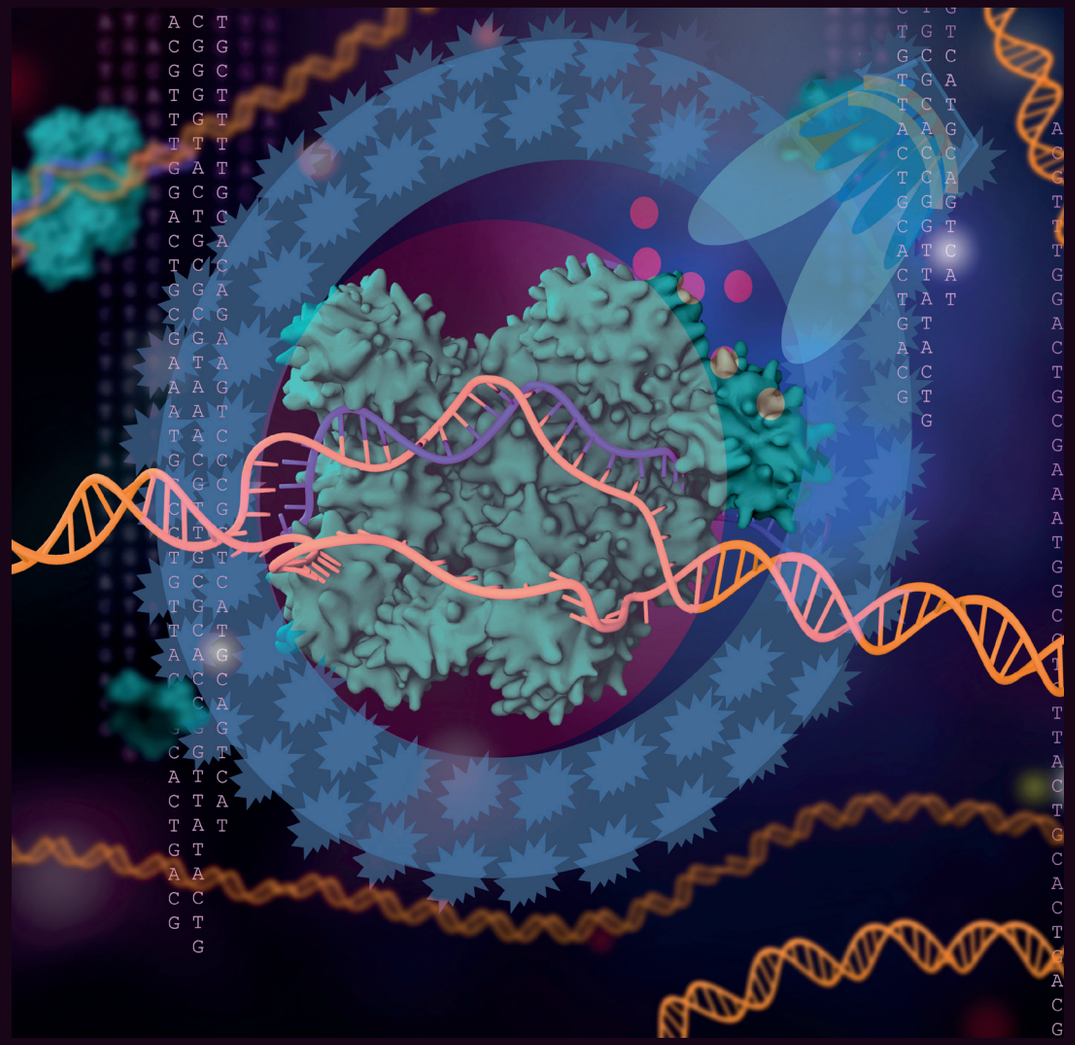

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 28 november 2018
klokke 11:15 uur

door

Catherin Yizet Marin Mogollon
geboren te Bogota (Colombia) in 1985

ISBN: 978-94-6182-919-1

The work presented in this thesis was performed at the Leiden Malaria Research Group, Department of Parasitology of the Leiden University Medical Center in the Netherlands.
Catherin Yizet Marin Mogollon was funded by PhD scholarship Colciencias-Colfuturo, (Colombia)

Layout and printing: Off Page, Amsterdam (www.offpage.nl).

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DEDICATION

This thesis is dedicated to my mother, Dennize Marin Mogollon,
who has made of me who I am;
To my grand mother, Edith Mogollon
For her unconditional love;
to my partner, love and best friend, Juan Carlos Alarcon,
who has cured my wounds and supported me in each adventure;
and to my son, Juan Daniel Alarcon,
for being my inspiration every day, and for making sense of my life.

Table of Contents

CHAPTER 1

Introduction

Malaria, the parasite and disease

Malaria is a vector-born disease of global health importance [1] with 216 million cases in 91 countries in 2016 resulting in around 445.000 deaths [2]. The greatest burden of malaria is in sub-Saharan Africa, where it takes the lives of more than 1,200 children each day [2]. Malaria is caused by a protozoan unicellular parasite, *Plasmodium*, which is transmitted by *Anopheles* mosquitoes. Five *Plasmodium* species are responsible for malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [3]. Most clinical cases are caused by *P. falciparum* and *P. vivax*, with *P. falciparum* being the deadliest [1, 3]. *P. falciparum* infections can cause severe anaemia, fever and organ damage, including cerebral complications; in contrast, *P. vivax* infections are usually not fatal but can be severe with recurrent clinical episodes of malaria associated with morbidity [4]. *P. malariae* and *P. ovale* infections are less well studied but the severity of illness caused by these parasites is similar to *P. vivax* malaria [4]. *P. knowlesi* is primarily a zoonotic infection encountered in Southeast Asia that can cause severe malaria [5].

The P. falciparum life cycle

Figure 1 depicts the life cycle of *P. falciparum*. A malaria infection in humans begins with the inoculation of *Plasmodium* sporozoites into the host dermis by the bite of an infected female *Anopheles* mosquito [4, 6]. The sporozoites can take 1-3 hours to exit from this site. Here they rely on gliding motility to penetrate a blood vessel, entering the blood stream and migrating to the liver [7]. Sporozoites recognise hepatocytes and infect these cells after the sporozoite become activated through a mechanism that involves interactions of host and parasite membrane proteins. Entry is gained by proteins that are released from the apical organs of the sporozoite, specifically the micronemes and rhoptries. Once hepatocyte infection is established, the parasite grows and divides in the next 2-10 days. These liver-stage (LS) or exo-erythrocytic forms (EEF) mature and release up to 40.000 merozoites per infected hepatocyte into the blood stream [3]. Once released into the circulation, the merozoites invade erythrocytes, where they grow, divide and form new merozoites, which upon release invade new red blood cells, initiating the repeated asexual replication cycles. All symptoms of malaria are associated with the blood-stage infection [8]. Within a red blood cell *P. falciparum* parasites progress over the course of 48 hours through the ring and the trophozoite stage before replicating into 8-32 merozoites at the schizont stage (schizogony) [1]. During the schizogony cycles in red blood cells, a proportion of parasites stop asexual division and undergo a developmental switch, initiating sexual development by development into male or female gametocytes (gametocytogenesis). These gametocytes mature through five defined stages over the course of 8-11 days [1, 9]. Once ingested by a mosquito the mature male and female gametocytes emerge from the red blood cell and rapidly produce gametes (gametogenesis), with the male gametocyte dividing into eight flagellated microgametes (exflagellation) and the female gametocyte developing into a single macrogamete. The female gamete is fertilised by

the male in the mosquito midgut, resulting in a diploid zygote, which elongates into an ookinete that penetrates the mosquito gut wall. The ookinete develops into an oocyst that undergoes cycles of replication to form several thousand sporozoites (sporogony), over a period of 9-12 days. These oocysts then burst to release the sporozoites that migrate to the salivary glands, resulting in mosquitoes that are infectious to humans [9, 10].

Malaria, the health problem

Malaria incidence and transmission depends on environmental suitability for local mosquito vectors, which includes altitude, climate, vegetation, and implementation of control measures. The intensity of the host-vector-host transmission depends on factors related to the parasite, the vector, the human host and the environment [11]. Currently, half of the world's population is at risk of malaria, with some population groups at higher risk of developing severe disease than others. These include infants, children under 5 years of age, pregnant women and immunosuppressed patients [2]. Early diagnosis and treatment reduces disease, prevents death and directly contributes to a reduction in malaria parasite transmission [4, 11]. However after considerable global success in malaria control over

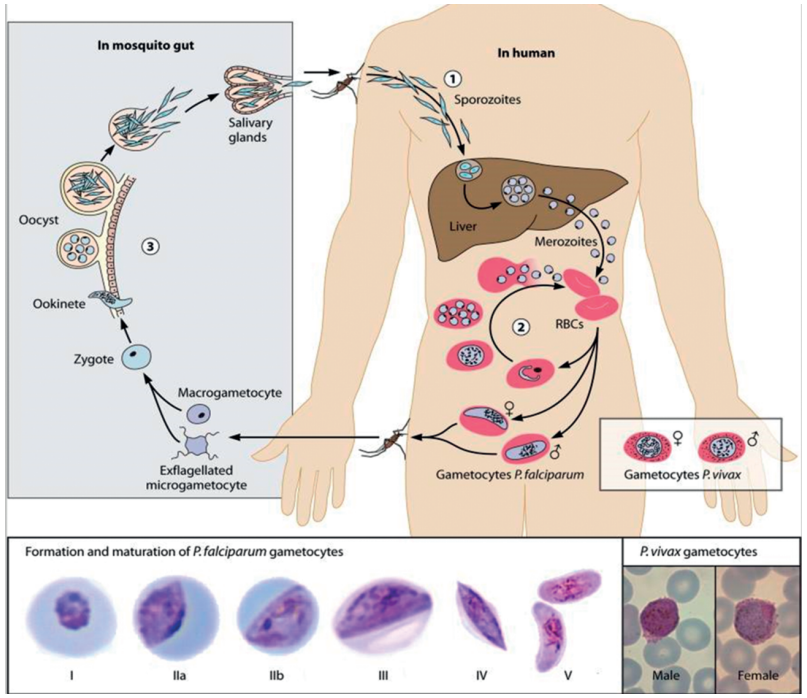

Figure 1. Schematic representation of the *P. falciparum* life cycle. Upper panel, *P. falciparum* development in mosquitoes (grey box) and in humans. Lower panel, the five developmental stages of *P. falciparum* gametocytes and mature *P. vivax* gametocytes. This image was taken from Bousema, T., et. al. (2011)[10].

the past 10-15 years, progress has now stalled according to the WHO World Malaria report 2017 [2]. A major problem is insufficient funding at both domestic and international levels, resulting in gaps in coverage of the use of insecticide-treated nets, antimalarial drugs, indoor residual spraying (IRS) with insecticides and other lifesaving tools [2]. Moreover, there is a global increase in resistance to front-line antimalarial drugs, such as chloroquine, sulfadoxine/pyrimethamine [12] and recently to artemisinin [13]. This highlights the need for novel drug and vaccine intervention programs against both the parasite and vector. In addition to novel insecticides and approaches to reduce transmission by mosquitoes, the identification of new classes of drugs as well as vaccines that target different stages of the parasites are required to protect the groups most at risk [2, 12] and to develop the most cost-effective means to prevent, eliminate and eradicate malaria [11].

Malaria, vaccine and drug development

Malaria vaccines are generally classified in different types of vaccines, defined by the different life-cycle stages of the parasite that are targeted by the vaccine. Specifically, (1) pre-erythrocytic vaccines, which induce antibodies and/or cell-mediated immune responses that block sporozoite invasion of hepatocytes or remove infected hepatocytes [11]; (2) blood-stage vaccines that are designed to block merozoite invasion of red blood cells or to eliminate infected red blood cells [12]; and (3) transmission blocking vaccines (TB vaccines) that generate antibodies that can block transmission of parasites in the mosquito by either blocking parasite fertilisation or zygote development. Pre-erythrocytic vaccines have been shown to prevent infection and can induce sterile protection against malaria. Blood-stage vaccines are likely to reduce the overall parasite burden in the blood and therefore reduce malaria symptoms and TB vaccines may be an effective means to reduce the spread of malaria within a population [12].

Currently, the most extensively tested vaccine candidate for prevention of *P. falciparum* malaria is RTS,S/AS01 (RTS,S; also known as Mosquirix), a pre-erythrocytic vaccine candidate [14]. This subunit vaccine targets the sporozoite and the infected liver cell and is based on the immunodominant antigen that covers the surface of the sporozoite, circumsporozoite protein (CSP). In RTS,S the CSP fragment is fused to hepatitis B virus surface antigen and administered with the adjuvant AS01 [14]. This is the only vaccine that has shown protective efficacy against clinical malaria in a Phase III clinical trial, but protective efficacy is modest and wanes over time and may be age dependent [15]. In vaccine development against *P. vivax* the CSP protein is also seen as an important vaccine target since evidence from pre-clinical and clinical studies has indicated that immune responses against *P. vivax* CSP play a role in mediating protection against *P. vivax* infections [16].

In addition to CSP, several other antigens of sporozoites and liver-stages have been identified as target antigens for subunit vaccines, for example CelTOS (cell traversal protein for ookinetes and sporozoites) and TRAP (thrombospondin-related adhesion protein). Recombinantly produced CelTOS of *P. falciparum*, a micronemal secreted-

protein, is one of the few vaccines that has recently entered clinical testing (NCT01540474 https://clinicaltrials.gov/). A viral-vectored sub-unit vaccine directed against TRAP fused to a multi-epitope string, demonstrated some protection against malaria infection in malaria-naïve adults [17].

Most research on the development of asexual blood-stage vaccines has focused on only a few antigens, for example merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1). Antibodies against both proteins correlate with naturally-acquired immunity in multiple epidemiological studies and vaccines targeting these antigens induced protective immune responses in preclinical studies in rodents [18]. However, although some studies performed in humans have shown some efficacy, no blood-stage vaccine has reached phase III testing [19]. It is important to consider the challenges that have faced the development of blood-stage vaccines, such as high levels of antigenic polymorphism and redundant pathways of invasion of red blood cells by merozoites. Different preclinical assays are used to predict the efficacy of blood-stages vaccines [19, 20]. The growth inhibition assay (GIA) is one of the most widely used functional assays to test interventions against asexual blood-stage development. In vaccine-based studies, blood-stage parasites are co-cultured with either control or test antibodies and the percentage of reduction in parasitemia is measured after a defined culture period [19].

For TB-vaccines, the leading target antigens include the ookinete surface protein Pf25 and the gametocyte/gamete antigens Pf48/45 and Pf230. Antibodies against these antigens perform well in inhibition of transmission in comparative preclinical studies, with functionality assessed by the standard membrane feeding assay (SMFA) using mosquitoes that are fed with cultured gametocytes in the presence of antibodies, whole serum or purified IgG [21]. The most advanced TB vaccine candidates are based on the antigens P48/45 of gametes and P25 of zygotes and ookinetes and recombinant vaccines of P25 of both *P. falciparum* and *P. vivax* have progressed into Phase I trials [22-24].

Despite three decades of testing different (recombinant) sub-unit vaccines, both in the clinic and the field, only modest protection against infection has been achieved [15, 25-27], which has renewed an interest in whole parasite-based vaccine approaches [28, 29]. It was first shown in rodent models of malaria that complete protection against infection could be obtained by vaccination using live attenuated sporozoites [30, 31]. Subsequently, sterile protection against malaria was also demonstrated in humans after immunization with *Plasmodium falciparum* sporozoites, either attenuated by radiation [32, 33] or administered under chemoprophylaxis [34]. A prerequisite for induction of protective immunity using sporozoite-based vaccines is that sporozoites retain their capacity to invade liver cells after administration. While the precise mechanisms of protection mediated by immunization with attenuated sporozoites remain unknown, T cells appear to be critical for protection and in particular $CD8^+$ T cells are thought to play a major role in eliminating infected hepatocytes [35]. The most advanced live-attenuated vaccine is based on irradiation-attenuated sporozoites (Irr-Spz), which is currently being evaluated both in the clinic and in field trials [36, 37]. In rodent models, immunization

with sporozoites of genetically-attenuated parasites (GAP) can induce similar or even better levels of protective immunity compared to Irr-Spz [35, 38]. Genetic attenuation of sporozoites has been achieved through the deletion of one or more genes that play a critical role during liver-stage development, resulting in complete arrest of parasite growth in the liver, thereby preventing a blood-stage infection after immunization with GAP sporozoites. Currently two *P. falciparum* GAP-based vaccines are undergoing clinical evaluation [38-42].

While the search for an effective vaccine against malaria remains a very active area of research, the most effective means to treat and prevent malaria remains the use of drugs [43]. However, resistance to available antimalarials continues to spread, including resistance to the widely used artemisinin-based combination therapies [44]. As multi-drug resistance spreads, there is an urgent need for new antimalarial agents to control malaria infections [43]. At the forefront of antimalarial development is the Medicines for Malaria Venture (MMV), a not-for-profit, public–private partnership (www.mmv.org). The current MMV portfolio contains many promising compounds at various stages of development [43]. New classes of antimalarial compounds have been identified in high-throughput screens of large compound libraries [45]. Most of such screens involve the exposure of different life cycle stages of *P. falciparum* parasites to the compounds and the measurement of inhibition of development. For example in short-term cultures of the blood-stages and determination of inhibition of blood-stage growth and multiplication. Such screening of large compound libraries requires highly reproducible and cost-effective assays that are amenable to automation and can be performed in a small culture volumes [43, 45].

Malaria elimination is likely to require a combination of interventions, including the generation, testing and implementation of new drugs and vaccines as well as new vector control strategies.

Genetically modified malaria parasites and their use in malaria research: the aim of the studies described in this thesis

In the mid-nineties, genetic modification to create permanent modifications in malaria parasite genomes was first described in the rodent malaria parasite *Plasmodium berghei* [46]. This technology was extended to other *Plasmodium* species, including the human malaria parasite *P. falciparum*, and was initially used for loss-of-function analyses to uncover the function of *Plasmodium* genes, including genes encoding potential vaccine candidate antigens (reviewed in [47, 48]). In addition to gene disruption and gene mutation, methodologies have been developed for creating malaria parasites that express 'foreign' genes from other organisms, so-called transgenic parasites. Among the first transgenic mutants were rodent malaria parasites (RMP) that were modified to express fluorescent and luminescent reporter proteins. These parasites have been used to visualize and analyse parasite growth and development *in vitro* and *in vivo*, and have been valuable tools to

analyse cellular and molecular aspects of malaria parasite biology (reviewed in [49-52]), and to study host-parasite interactions and pathology [53-58]. In addition, transgenic rodent parasites have been used to develop and evaluate vaccines (reviewed in [59]). For example, chimeric RMP expressing *P. falciparum* or *P. vivax* antigens have been used to directly evaluate human malaria vaccines before their advancement to clinical testing.

Transgenic parasites expressing fluorescent or luminescent reporter proteins have also been created in the human parasite *P. falciparum* and the primate parasite *P. cynomolgi*. These transgenic parasites have been exploited in screening assays to measure (inhibition of) parasite growth at different points of the parasite life cycle. GFP- and luciferase-expressing *P. falciparum* parasites have been used *in vitro* to examine the effect of drugs and other inhibitors on blood-stage growth and on gametocytes [51, 60-63] and fluorescent *P. cynomolgi* parasites have been generated to screen for compounds that target the hypnozoite stage in the liver [64].

For RMP, the availability of transgenic parasite lines expressing different reporter proteins under the control of stage-specific or constitutive promoters has been of great benefit to research of parasite gene function and on research focused on evaluation of novel drugs and vaccines. The availability of similar *P. falciparum* transgenic reporter lines would open up possibilities to perform these studies directly with the human malaria parasite. For example, strongly fluorescent liver-stage *P. falciparum* parasites could create possibilities for enriching infected hepatocytes by flow-sorting methods, which would aid identification of novel vaccine targets, or mCherry-expressing *P. falciparum* parasites could be used to analyse parasite interactions with host cells (e.g. sporozoites with cells of the immune system or hepatocytes). Increasingly, cell-cell interactions in culture are examined using transgenic host cells expressing, for example, green fluorescent protein; therefore, the availability of transgenic *P. falciparum* parasites expressing different fluorophores can boost such studies. The creation of transgenic RMP expressing more than one transgene has permitted more elaborate and intricate studies on parasite biology and immunity. For example, fluorescent parasites that also express the immunological reporter antigen ovalbumin have been used to better understand how parasite antigens induce protection by examining interactions of infected hepatocytes with anti-OVA OT-1/2 T-cells [55]. The creation of transgenic parasites stably expressing multiple transgenes is dependent on the presence of multiple suitable target loci in the parasite genome, which can be modified without altering parasite growth and development. In *P. falciparum* the *p47* gene locus has most frequently been used to introduce transgenes [65, 66]. Therefore, the identification of other suitable 'neutral' genomic loci would greatly aid in the generation of mutants expressing multiple transgenes.

The main aim of the studies described in this thesis was to develop novel CRISPR/Cas9 methodologies to improve *P. falciparum* transgenesis. This was done in order to create novel transgenic reporter parasites that can be used to analyse host-pathogen interactions and for anti-malarial drug and vaccine research. We first focused on improving CRISPR/Cas9 gene editing technology and on introducing transgenes into the *P. falciparum* genome using a new potential 'neutral' locus. Using this improved CRISPR/Cas9 methodology, transgenic *P. falciparum* parasites were created that either express fluorescent-luminescent reporters or express a major vaccine candidate from the other major human malaria parasite, *P. vivax*. The outline of the different studies is explained in more detail below, as well as the rationale for the different approaches taken to generate these transgenic parasites.

Outline of this study

In **Chapter 2** we provide a review on the use of transgenic malaria parasites in the development of malaria vaccines targeting different stages of the parasite life-cycle. While transgenic *P. falciparum* parasites have been used in studies to evaluate both antimalarial drugs and vaccines, the majority of the studies use transgenic RMP, for which a greater number of techniques is available to genetically modify and examine the parasite throughout the complete life cycle.

Improved CRISPR/Cas9 genetic modification of P. falciparum (Chapter 3)

For rodent malaria parasites (RMP) efficient technologies have been developed for stably introducing transgenes into the parasite genome and efficient and rapid methods are available for the generation of transgenic reporter parasites that do not contain drug-selectable markers [67, 68]. Such 'marker-free' parasites make it considerably easier to further genetically modify transgenic parasites; moreover, they can be used for drug-sensitivity testing, as possible interference from an introduced drug-selection marker is absent. For RMP, a variety of transgenic reporter parasite lines have been generated in multiple strains of three different *Plasmodium* species [69]. In comparison to RMP, the technologies to genetically modify the human malaria parasite *P. falciparum* are much less efficient [48]. Traditional approaches to engineer the *P. falciparum* genome have been hampered by the limited methods available and transfection inefficiencies for introducing exogenous DNA into the parasite genome. Also, the limited number of drug-selectable markers restricts genetic engineering of *P. falciparum*; for example, performing sequential genetic manipulations in the same parasite line. Several technologies have been developed for the removal (re-cycling) of drug-selectable markers from the modified parasite genome, specifically using FLP and Cre recombinases [70, 71]. However, the application of these techniques is time consuming, as it can take 4-5 months to generate cloned 'marker-free' genetically modified parasites. The RNA-guided CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system has transformed genome editing in a wide variety of organisms [72]. This powerful genome editing technique has also been applied to *P. falciparum* and provides efficient methods to manipulate the parasite's genome, such as site-directed mutagenesis, gene disruption and the introduction of transgenes [73, 74]. Generation of *P. falciparum* transgenic reporter parasites would benefit from the availability of standard CRISPR/Cas9 plasmids that

permit the rapid introduction of different transgenes into the parasite genome without permanently integrating a drug-selectable marker cassette. Currently, no cloned reporter lines have been published that are drug-selectable marker free.

In **Chapter 3** we describe studies aimed at improving CRISPR/Cas9 genetic modification for introduction of transgenes into the genome of *P. falciparum* without the inclusion of a drug-selectable marker cassette. We describe the generation of transgenic parasites expressing GFP under control of different *P. falciparum* promoter regions that were selected based on their high (and constitutive) expression in different life cycle stages. The GFP-expression cassettes were introduced into the genome in the *p230p* gene locus, which we predicted to be a 'neutral' locus. We examined and compared the GFP-reporter expression of the three novel transgenic lines at different points during blood-stage development. However, disruption of the *P230p* locus unexpectedly resulted in parasites that could not infect and develop in mosquitoes. The phenotype of the 'gene-deletion' mutants in mosquitoes and the potential role of the P230p protein in mosquito development is described in more detail in **Chapter 4**.

Characterization of P. falciparum mutant (reporter) lines lacking P230p expression (Chapter 4)

In *P. falciparum* the *p47* gene locus has been most frequently used to introduce transgenes into the *P. falciparum* genome [65, 66]. We initially had selected the *p230p* gene locus to introduce transgenes as an alternative to the *p47* gene, since the P47 protein has been shown to be important for limiting the host-defence responses against the parasite in mosquitoes [75, 76] . Consequently, *P. falciparum* parasites lacking P47 expression are less efficiently transmitted by some strains of *Anopheles* mosquitoes, as they have a decreased capacity to escape the mosquito immune response. In two rodent *Plasmodium* species the male-specific P230p protein appears to be dispensable throughout the parasite's complete life cycle [77-79]. *P. berghei* and *P. yoelii* mutants lacking expression of P230p can develop in the vertebrate host and in the mosquito vector without a discernible phenotype and *p230p* knock-out parasites manifest a wild type parasite phenotype. Consequently, as P230p is non-essential, the *p230p* gene is the locus most frequently used to introduce additional transgenes into rodent malaria parasite genomes [78].

P230p and P47 belong to the s48/45 domain 6-cysteine (6-cys) family of *Plasmodium* proteins, a small family with 14 members that show stage-specific expression throughout the parasite life cycle and most members localize at the parasite surface [80]. Most members have critical roles in parasite development, either in the vertebrate host or in the mosquito vector, and several members are leading targets for malaria vaccines. These include vaccine antigens that target parasites in the mosquito, the so called transmission blocking vaccines, i.e. P48/45 and P230 which are paralogs of P47 and P230p, respectively [81-83]. In both *P. berghei* and *P. falciparum* P47 is specifically expressed in female gametocytes/gametes and is located on the surface of female gametes, zygotes and ookinetes [84]. P47 is important in protecting ookinetes from the mosquito's complement-like immune

response in both rodent and human malaria species [76, 85, 86]. In addition, *P. berghei* P47 plays an essential role in the attachment and recognition of the female gamete by the male gamete [77, 85]. In contrast, *P. falciparum* P47 does not play such a crucial role in gamete fertilization [84].

In **Chapter 4** we characterise in more detail some of the transgenic reporter lines we have described in **Chapter 3**, where the reporter cassette had been introduced into the *P230p* locus. Specifically, we examine the phenotype of these parasites during sexual blood-stage development and early mosquito stages.

Generation of a transgenic P. falciparum parasite line expressing fluorescent and luminescent protein in different life cycle stages (Chapter 5)

For RMP the availability of transgenic reporter parasites expressing different fluorescent and luminescent proteins under the control of stage-specific or constitutive has been of great benefit to research of parasite gene function and research focused on evaluation of novel drugs and vaccines. Such transgenic reporter lines for *P. falciparum* would benefit research where *P. falciparum* parasites are used (see the sections above).

In **Chapter 3** we described studies to test different *P. falciparum* promoter-GFP expression cassettes. These studies were performed in order to generate parasite lines that express fluorescent proteins at high levels throughout the complete life cycle. However, given that the insertion of transgenes into the *P. falciparum p230p* locus resulted in parasites that could no longer infect mosquitoes (**Chapter 3 and 4**), we reverted to using the standard 'neutral' *p47* gene locus for introduction of a novel reporter expression cassette. In **Chapter 5**, we describe the creation and evaluation of a reporter line that expresses a fusion of mCherry and luciferase driven by the promoter of the *etramp10.3* gene and examine these transgenic parasites in blood- and liver-stage cultures, as well as in mosquitoes. We selected this promoter because *etramp10.3* has structural similarity to the *uis4* gene of RMP and both genes have the same syntenic genomic location. In transgenic RMP lines the promoter of the *uis4* gene has been used to drive expression of multiple transgenes specifically in sporozoites and liver-stages, such as genes encoding mCherry, ovalbumin or human malaria proteins [87-93]. The *uis4* gene is highly transcribed in sporozoites and liver-stages and encodes a parasitophorous vacuole membrane (PVM) protein that surrounds the parasite in the infected hepatocyte [87]. Evidence has been presented for expression of *etramp10.3* in *P. falciparum* sporozoites and in blood- and liver-stages where the protein is located at the PVM, similar to the PVM location of UIS4 in liver-stages of RMP [94]. We chose to generate an mCherry-expressing *P. falciparum* line, as it could be used to visualise interactions of *Plasmodium* sporozoites with host-cells (e.g. immune cells or hepatocytes) which are often labelled with green fluorescent proteins. Moreover, we fused the mCherry gene to the gene encoding firefly luciferase as luciferase expression can be used to quantify parasite numbers (e.g. sporozoites and liver-stages) using simple and sensitive luminescence assays [62, 95, 96].

Generation of chimeric P. falciparum parasites that express vaccine candidate antigens from the human malaria parasite, P. vivax (Chapter 6)

Testing the next generation of *P. falciparum* vaccines and vaccine formulations is greatly aided by being able to perform immunization studies in people followed by malaria-parasite challenge in controlled human malaria infections (CHMI) [97-100]. CHMI studies have increased the speed of vaccine evaluation by using well-controlled early-phase proof-of-concept clinical studies. Such studies facilitate the down-selection of vaccine candidates and the identification of those candidates most suitable for further evaluation in more expensive and complex phase II/III trials in areas where malaria is endemic.

Although recently CHMI has also been developed for *P. vivax* [101] and has been applied to assess pre-erythrocytic vaccine candidates [102, 103], the use of *P. vivax* CHMI to rapidly screen different *P. vivax* vaccines is limited because of the lack of methods to continuously propagate *P. vivax* blood-stages in culture and to produce gametocytes *in vitro* that can be used to infect mosquitoes to produce sporozoites for challenge infections [101]. Therefore, *P. vivax* CHMI is dependent on sporozoites that have been obtained from mosquitoes fed on infected patients [101]. Moreover, *P. vivax* sporozoites can produce hypnozoites, dormant forms that can persist in the liver for prolonged periods, which requires safe and effective means to clear these forms from the liver in CHMI studies [101, 104].

In preclinical evaluation of vaccines, chimeric rodent malaria parasites (chimeric RMP) expressing *P. falciparum* and *P. vivax* pre-erythrocytic antigens have been used to analyse protective immune responses induced by *P. vivax* or *P. falciparum* vaccines *in vivo* in mice. These chimeric RMP have been used to assess the protective immune responses induced by vaccination that influence sporozoite invasion of hepatocytes both *in vitro* and *in vivo,* and the removal of infected hepatocytes *in vivo* [59]. For example, chimeric RMP have been generated where the endogenous *csp* gene has been replaced either with *P. falciparum csp* or different *P. vivax csp* alleles. These chimeric parasites produce sporozoites that are infectious to rodent hepatocytes *in vivo* and human hepatocytes in culture [59].

Based on studies with chimeric rodent parasites, we reasoned that the availability of chimeric *P. falciparum* parasites that express *P. vivax* antigens would open up possibilities to analyse protective immune responses induced by vaccination using *P. vivax* antigen-based vaccines in CHMI, bypassing the need for *P. vivax* parasite production and measures to ensure that *P. vivax* hypnozoites are removed. As a proof of concept we explored in **Chapter 6** the possibility to create, using CRISPR/Cas9 gene editing methodologies, two chimeric *P. falciparum* parasites where the gene encoding circumsporozoite protein (CSP), was replaced by *csp* genes of *P. vivax*. CSP is the major protein of the sporozoite surface [14, 109] and plays a critical role both in sporozoite formation and in sporozoite invasion of mosquito salivary glands and liver cells of the host [105-108]. CSP is the target antigen of the most advanced *P. falciparum* malaria vaccine (RTS,S) and is also an important vaccine target for *P. vivax* [110, 111].

In **Chapter 7** the results of the studies described in **Chapters 3-6** are summarized and discussed, including a discussion on the use of transgenic *P. falciparum* parasites in research aimed at developing novel drugs and vaccines

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CHAPTER 2

The use of transgenic parasites in malaria vaccine research

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Expert Review of Vaccines, 2017 Jul; 16(7): 1-13

Abstract

Introduction

Transgenic malaria parasites expressing foreign genes, for example fluorescent and luminescent proteins, are used extensively to interrogate parasite biology and host-parasite interactions associated with malaria pathology. Increasingly transgenic parasites are also exploited to advance malaria vaccine development.

Areas Covered

We review how transgenic malaria parasites are used, *in vitro* and *in vivo*, to determine protective efficacy of different antigens and vaccination strategies and to determine immunological correlates of protection. We describe how chimeric rodent parasites expressing *P. falciparum* or *P. vivax* antigens are being used to directly evaluate and rank order human malaria vaccines before their advancement to clinical testing. In addition, we describe how transgenic human and rodent parasites are used to develop and evaluate live (genetically) attenuated vaccines.

Expert Commentary

Transgenic rodent and human malaria parasites are being used to both identify vaccine candidate antigens and to evaluate both sub-unit and whole organism vaccines before they are advanced into clinical testing. Transgenic parasites combined with *in vivo* pre-clinical testing models (*e.g.* mice) are used to evaluate vaccine safety, potency and the durability of protection as well as to uncover critical protective immune responses and to refine vaccination strategies.

Keywords

Plasmodium, Malaria, Transgenic, Vaccine, Reporter, GAP, Chimeric.

Introduction

In the mid-nineties genetic modification to create permanent modifications in malaria parasite genomes was first described in the rodent malaria parasite *Plasmodium berghei*[1]. This technology was extended to other *Plasmodium* species, including the human malaria parasite *P. falciparum,* and was initially used for loss-of-function analyses to uncover the function of *Plasmodium* genes, including genes encoding potential vaccine candidate antigens (reviewed in[2, 3]). In addition to gene-disruption and gene-mutation, methodologies have been developed to create malaria parasites that express 'foreign' genes from other organisms, so called transgenic parasites. Amongst the first transgene mutants were rodent malaria parasites that expressed fluorescent and luminescent reporter proteins. These parasites have been used to visualize and analyze parasite growth and development *in vitro* and *in vivo* and have been valuable tools to analyze cellular and molecular features of malaria parasite biology (reviewed in [4-7]). Transgenic rodent parasites have also been used to provide mechanistic insights into host-parasite interactions that regulate host (immune) responses to infection or those that mediate malarial pathology [8-13].

Transgenic parasites expressing fluorescent or luminescent reporter proteins have been created in rodent malaria species, the human parasite *P. falciparum* and the primate parasite *P. cynomolgi*. These parasites have been exploited in screening assays to measure (inhibition of) parasite growth at different points of the parasite life-cycle. Fluorescent and luminescent *P. falciparum* parasites have been used *in vitro* to examine the effect of drugs and other inhibitors on blood stage growth and on gametocytes[6, 14-17]and fluorescent *P. cynomolgi* parasites have been generated to screen for compounds that target the hypnozoite stage in the liver[18]. Transgenic fluorescent and luminescent rodent parasites have been used in *in vitro* screening assays to test inhibitors that target parasite development in the blood and liver [6, 19-22].

In addition to measuring growth inhibition *in vitro*, transgenic rodent parasites have been used to examine the impact of drug or vaccine interventions *in vivo*, where inhibition of parasite development is measured as the reduction of reporter signal(mostly luminescent) in organs of the treated (compared to unimmunized/untreated) rodent host[6, 17, 19, 22, 23]. As the life-cycle and basic biology of rodent and human *Plasmodium* parasites are very similar and since the vast majority of genes within their genomes are conserved [24], transgenic rodent parasites are frequently used to evaluate protective immunity against candidate *Plasmodium* antigens *in vivo* and are used to assess different vaccine delivery platforms and vaccination regiments. Several of these studies have been conducted in different inbred mice strains that exhibit different, often polarized, immunological responses to infection. Transgenic rodent parasites have been used in preclinical studies to examine protective immune responses to pre-erythrocytic (sporozoite and liver stage) vaccines (see **Section 2**).

More recently transgenic rodent parasites have been generated that express proteins of the human *Plasmodium* species *P. falciparum* and *P. vivax*. These so-called 'chimeric' parasites have been used to evaluate the (*in vivo*) action of drugs against human *Plasmodium* protein targets [25, 26], to study malaria pathology during pregnancy, *in vivo* [27] and to evaluate the protective efficacy of vaccines that target human *Plasmodium* antigens (reviewed in [28-30] and see **Table 1**). In these vaccine studies, mice are immunized with *P. falciparum or P. vivax* antigens and subsequently challenged with chimeric rodent parasites expressing the cognate *P. falciparum* or *P. vivax* antigens. Such chimeric parasites permit an *in vivo* immunological evaluation of novel target *Plasmodium* antigens and vaccination strategies and can indicate the magnitude and type of protective immune response induced. This knowledge can be used to down-select from candidate antigens under consideration before proceeding to clinical studies [31].

Lastly, genetic modification of rodent and human malaria parasites has also been used to generate parasites that arrest in the liver. These parasites can provoke strong protective immune responses in the host and are therefore being evaluated as live, attenuated vaccines [32-34].Many gene-deletion rodent parasites have been tested in rodents for growth-arrest in the liver and for their capacity to induce potent protective immune responses. These so called GAPs have been created in transgenic reporter lines, which simplifies the *in vivo* evaluation of both their safety and protective efficacy. In order to generate completely safe GAP vaccines, GAPs must be generated that completely arrests in the liver. Consequently, multiple gene-deletions in the same GAP are considered necessary, each governing independent but essential processes during liver stage development. Therefore, in order to generate and test a *P. falciparum* GAP in human test subjects, large scale screening of single and multiple gene-deletion mutants in rodents is necessary to identify suitable genes for deletion in *P. falciparum*.

In this review we describe the use of transgenic malaria parasites and their use as preclinical evaluation tools to measure vaccine efficacy and immune responses after vaccination. We describe: (i) transgenic rodent and human parasites that express reporter proteins that have been used to evaluate immunogenicity of vaccine antigens and vaccine efficacy; (ii) the use of transgenic chimeric rodent parasites, expressing antigens of *P. falciparum* or *P. vivax*, to compare immunogenicity of vaccines and vaccine strategies; and (iii) the use of transgenic parasites to identify and evaluate genetically attenuated parasite(GAP) vaccines and to examine immunological correlates of protection after vaccination *in vivo*.

Transgenic parasites expresing reporter proteins

transgenic rodent and human malaria parasites that express fluorescent and luminescent reporter proteins have been used in screening assays to efficiently and rapidly measure inhibition of parasite growth at different points of the parasite life-cycle [6, 17, 22, 35].

Table 1. Transgenic rodent and human malaria parasites used in malaria vaccine research

Transgenic rodent malaria parasites (RMP) expressing reporter proteins				
Reporter	**Remarks**			
Fluorescent proteins (e.g. GFP, mCherry)	A number of RMP expressing different fluorescent reporter proteins have been used to quantify parasite growth of different life cycle stages and to analyze interactions between infected cells and immune factors (see **Section 2** for references)[a]			
Luminescent proteins (e.g. luciferase)	A number of different luminescent reporter RMP have been generated that have been used to quantify parasite growth of different life cycle stages, both *in vitro* and *in vivo* (see **Sections2**and **4** for references)[a]			
Ovalbumin (OVA)	Several OVA-expressing RMP have been used to analyze interactions of the parasite with the host immune system (see **Sections2**and **4** for references)[a]			
Transgenic *P. falciparum* parasites expressing reporter proteins				
Reporter	**Remarks**			
GFP	GFP-expressing *P. falciparum* parasites have been used in GAI assays [16]			
Luciferase	Luminescent *P. falciparum* parasites have been used to quantify inhibition of oocyst production in SMFA assays [14]			
Chimeric rodent malaria parasites expressing human *Plasmodium*[b] proteins				
Protein product	***P. falciparum*/ *P. vivax* gene**	**Remarks**	**RMgm ID**	**Ref**
*Pf*LSA-1	PF3D7_1036400	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in *Pb* (ANKA)	#1314	[31]
*Pf*LSA-3	PF3D7_0220000	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in *Pb* (ANKA)	#1315	[31]
*Pf*CelTOS	PF3D7_1216600	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1310	[31]
*Pf*UIS3 (ETRAMP13)	PF3D7_1302200	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1311	[31]
*Pf*LSAP1	PF3D7_1201300	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1308	[31]
*Pf*LSAP2	PF3D7_0202100	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1312	[31]
PfETRAMP5	PF3D7_0532100	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1309	[31]
*Pf*Falstatin	PF3D7_0911900	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1313	[31]
*Pf*CSP	PF3D7_0304600	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1316	[31]
*Pf*TRAP	PF3D7_1335900	**Additional copy**; *Pf* (NF54) gene under the control of *Pbuis4* promoter; in*Pb* (ANKA)	#1317	[31]
*Pf*UIS3/ *Pf*TRAP	PF3D7_1302200 PF3D7_1335900	**(2) Additional copies**; *Pf* (NF54) genes under the control of *Pbuis4* promoter; in *Pb* (ANKA)	#4076	[76]
*Pf*CSP/ *Pf*TRAP	PF3D7_0304600 PF3D7_1335900	**(2) Additional copies**; *Pf* (NF54) genes under the control of *Pbuis4* promoter; in*Pb* (ANKA)		[95]

Table 1. (continued)

Protein product	*P. falciparum*/ *P. vivax* gene	Remarks	RMgm ID	Ref
*Pf*CSP	PF3D7_0304600	**Replacement copy**; *Pb* (ANKA)*csp* replaced by *Pf*(Wellcome strain) *csp*, full-length *Pbcsp* promoter & 302bp *Pbcsp*3'UTR. Reduced sporozoite production	#69	[73]
*Pf*CSP	PF3D7_0304600	**Replacement copy;** *Pb* (ANKA) *csp* replaced by *Pf*(NF54) *csp* under control of endogenous *Pbcsp* promoter and 3'UTR; No drug selectable marker. Normal sporozoite production and infectivity	#4110	
*Pf*CSP	PF3D7_0304600	**Replacement copy;***Py* (17XNL) *csp* replaced with *Pf* (3D7) *csp*. Human DHFR selectable marker. *Pbhsp70* 3'UTR Normal sporozoite production and infectivity	#1442	[96]
*Pf*TRAP	PF3D7_1335900	**Replacement copy;***Pb* (ANKA)*trap* replaced by *Pf*(NF54) *trap* under control of endogenous *Pbtrap* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity	#4112	
*Pv*TRAP	PVP01_1218700	**Replacement copy;***Pb* (ANKA) *trap* replaced with *Pv* (Sal-1) *trap*. No selectable marker. Normal sporozoite production and infectivity	#1103	[97]
*Pv*25	PVX_111175	**Replacement copy;** *Pb25* and *Pb28* replaced with *Pv 25; in Pb (ANKA)*	#222	[49]
*Pf*25	PF3D7_1031000	**Replacement copy;** *Pb25* and *Pb28* replaced with *Pf25; in Pb (ANKA)*	#273	[50]
*Pf*CelTOS	PF3D7_1216600	**Replacement copy;** *Pb* (ANKA) *celtos* replaced by *Pf* (NF54) *celtos* under control of endogenous *Pbceltos* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity	#4066	[74]
*Pv*CSP (VK210)	PVX_119355	**Replacement copy;** *Pb* (ANKA) *csp* replaced by *Pv*VK210 *csp* under control of endogenous *Pbcsp* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity		[77]
*Pv*CSP (VK247)	PVX_119355	**Replacement copy;** *Pb* (ANKA) *csp* replaced by *Pv* VK247 *csp* under control of endogenous *Pbcsp* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity		[77]
*Pv*CelTOS	PVX_123510	**Replacement copy;** *Pb* (ANKA) *celtos* replaced by *Pvceltos* under control of endogenous *Pbceltos* promoter and 3'UTR; No drug selectable marker Normal sporozoite production and infectivity	#4111	[75]
Rodent malaria parasites expressing HMP-RMP fusion proteins[b]				
CSP	PF3D7_0304600	The repeat region of *Pb*(NK65) *csp* is replaced with the *Pf* (7G8) *csp* repeat region.	#76	[98]

Table 1. (continued)

Protein product	*P. falciparum*/ *P. vivax* gene	Remarks	RMgm ID	Ref
MSP1	PF3D7_0930300	The *Pb* (ANKA) *msp-1*_19 C-terminal replaced with the *Pf* (D10) *msp-1*_19 C-terminal	#201	[78]
MSP1	PF3D7_0930300	The*Pb*(ANKA) *msp-1*19 C-terminal replaced with the *Pf* (FCC1/HN) *msp-1*_19 C-terminal	#330	[99]
CSP (VK210)	PVX_119355	The repeat region of *Pb* (ANKA) *csp* is replaced with the *Pv* (210) csp repeat region.	#906	[100]
CSP (VK210)	PVX_119355	The repeat region of*Pb* (ANKA) *csp* is replaced with (part of) *Pv* (210) *csp* gene	#1104	[47]
CSP (VK247)	PVX_119355	The majority of *Pb* (ANKA) *csp* gene is replaced with *Pv* (247) *csp;* the fusion gene retains *Pb* signal sequence (1-20aa) and *Pb* GPI anchor sequence (372-395aa)	#1443	[101]
P25	PVX_111175	The *Pb* (ANKA)*25* and *28* genes replaced with a fusion of *Pv*25 and *Pb* 25	#223	[49]
VAR2CSA	PF3D7_1200600	A synthetic *Pf* 3D7 DBL1X-6ε gene (*var2csa*) fused to *Pb* (ANKA) *fam-a*	#1436	[27]
Genetically Attenuated Parasites (GAPs)				
See **Section 4**for details (and references) of transgenic parasites used to generate and test GAP vaccines				

[a]For full list of transgenic reporter parasites generated in RMP see the **RMgm Database** www.pberghei.eu
[b]*Plasmodium* species abbreviations: *Pf - P. falciparum*; *Pv- P. vivax*; *Pb- P. berghei*; *Py- P. yoelii*

These assays have been used to identify and characterize anti-*Plasmodium* drugs and small molecule inhibitors, as well as vaccines targeting parasite development at different points of the life-cycle. Transgenic parasites expressing fluorescent or luminescent proteins have been generated in three RMP, *P. berghei*, *P. yoelii* and *P. chabaudi*. For *P. berghei* and *P. yoelii* a number of transgenic lines exist that express different reporter proteins such as GFP, mCherry or luciferase (or fusions thereof). Most of these lines express these proteins under control of *Plasmodium* promoters of constitutively expressed *Plasmodium* genes (often housekeeping genes), which creates parasites that can be visualized and quantified throughout the complete life cycle (**Figure 1A,B)**. Frequently used promoter regions of RMP genes include *elongation factor 1-ap*ha (*eef1*α), *dihydrofolatereductase-thymidylate synthase* (*dhfr-ts*) or *heat shock protein 70* (*hsp70*). Information on all published RMP transgenic lines can be found in the RMgm database of genetically modified rodent parasites (www.pberghei.eu).

Different assays have been developed to quantify parasite growth using reporter parasites. To test the effect of inhibitors on blood and liver stage growth, simple and rapid assays exist that can quantify parasite numbers in blood samples, infected hepatocytes or in other tissues. For example flow cytometric based assays counting GFP (or mCherry)

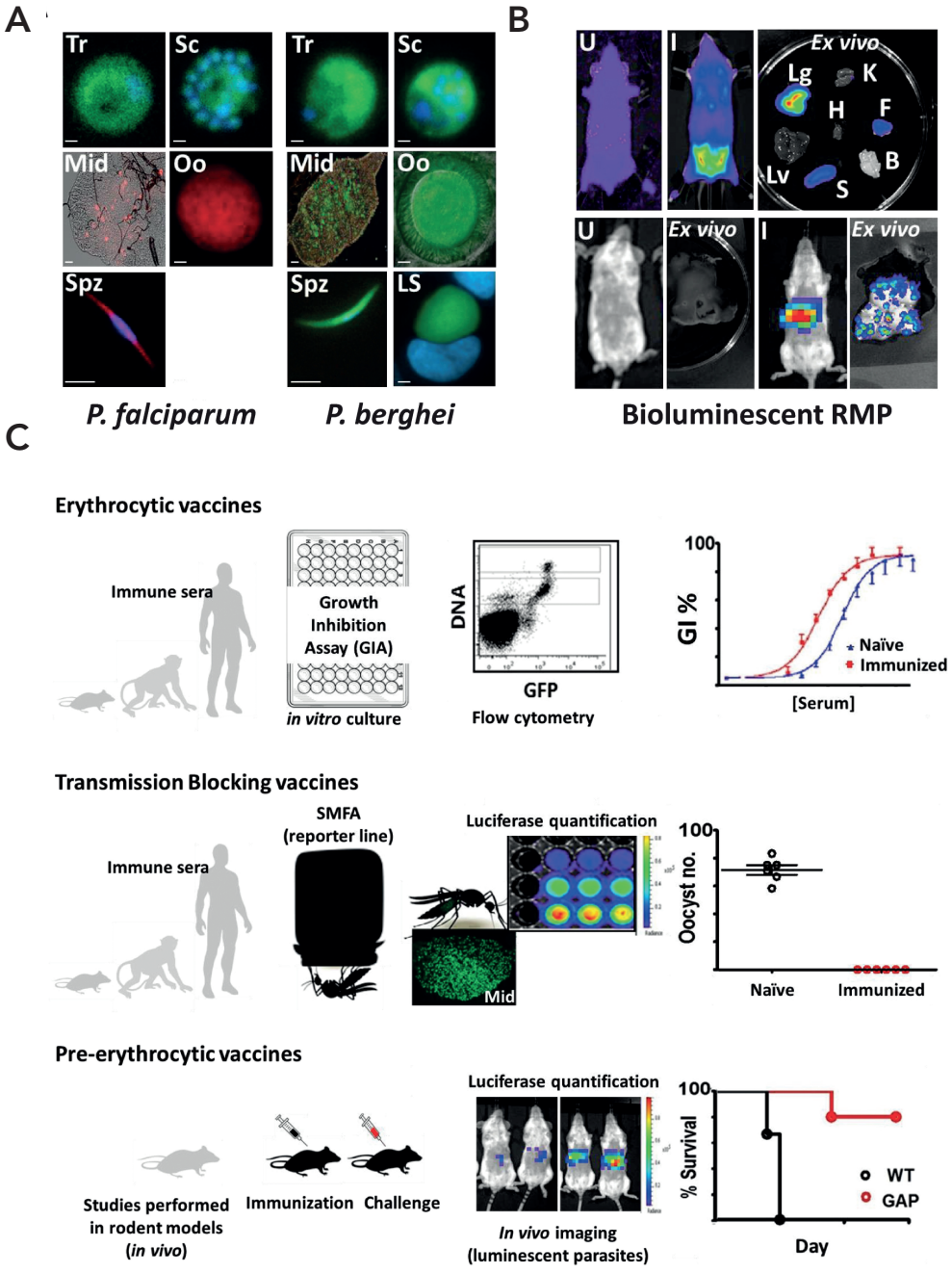

Figure 1. The use of transgenic reporter parasites in malaria vaccine research. **A**. Representative fluorescent images of different life cycle stages of *P. falciparum* and *P. berghei* (mCherry and GFP) reporter parasites. Blood stage trophozoites (Tr); schizonts (Sc); dissected infected mosquito midguts (Mid) with mature oocysts (Oo); salivary gland sporozoites (Spz); *P. berghei* liver stage schizont (LS). Host and parasite DNA are stained with Hoechst or DAPI (blue). **B**. Representative rainbow images of luminescence intensity in blood (upper panels) or liver (bottom panels) of live mice either uninfected

(U) or infected (I) with luminescent reporter parasites. Parasite density (luminescence intensity) can also be determined in extracted tissue (ex vivo); lungs (lg), kidney (K), adipose/fat tissue (F), liver (Lv), spleen (S), brain (B) and heart (H). Bottom panel shows luminescence in extracted livers of infected and uninfected mice, 48 h after infection with sporozoites. **C**. Schematic representations showing the use of transgenic reporter parasites in assays to determine efficacy of erythrocytic, transmission blocking (TB) and pre-erythrocytic (sporozoite and liver stage) vaccines. Erythrocytic Vaccines: The inhibitory activity of sera from (semi) immune individuals or purified immunoglobulins from vaccinated animals/people on parasite invasion and growth in red blood cells are frequently determined in Growth Inhibition Assays (GIA). GFP expressing *P. falciparum* parasites have been used in GIA where inhibition of parasite growth was determined by measuring parasitemia by flow cytometry. Transmission Blocking Vaccines: The standard membrane-feeding assay (SMFA) is a well-established method to evaluate the activity of antibodies/serum against human malaria parasites in the mosquito, mainly quantified by determination of oocyst production. A transgenic reporter *P. falciparum* line expressing luciferase have been used in SMFA to quantify oocyst production in mosquitoes, thus eliminating the need for mosquito dissections. Pre-erythrocytic (sporozoite and liver stage) Vaccines: Assays employing luciferase-expressing RMP have been developed to visualize and quantify liver stage development. Quantification of parasite liver loads by real time imaging has been performed in vaccinated and unvaccinated mice that have been challenged with luminescent parasites that either only express luciferase (e.g. in GAP studies; **Section 4**) or also express human malaria proteins (e.g. in studies on human malaria vaccines; **Section 3**).

positive parasite-infected red blood cells [20, 36, 37](**Figure 1A**) or quantification of luminescence signals to determine parasite numbers or parasite loads in blood, liver or other organs [19, 21](**Figure 1B**). Infecting mice with defined numbers of luciferase expressing parasites and subsequent quantifying parasite loads (luminescence signal) in the liver by real time imaging of live mice is frequently used to establish the *in vivo* effect of either inhibitors and vaccines on liver stage development [6, 17, 22, 23]. Bioluminescence imaging is simple to execute and can be used to monitor the course of an infection without sacrificing the animal [19] (**Figure 1B**). This reduces the number of animals required for experimentation because multiple measurements can be made in the same animal over time that also minimizes the effects of biological variation. In addition, since imaged mice do not have to be sacrificed, additional features of parasite development can be established, for example characteristics of the ensuing blood stage infection such as the prepatent period, *i.e.* the duration between sporozoite infection and a microscopically detectable blood infection. Bio-luminescence imaging is a proven and sensitive method to measure parasite liver loads in mice, even after infection with low sporozoite doses. It has been shown that parasite liver loads can still be determined even after inoculation of 1-10 sporozoites and that *in vivo* imaging quantification of parasite loads correlates very well with qPCR quantification methods [38]. The sporozoite doses used in different studies vary according to the vaccines being tested. Specifically, when examining potential GAP vaccines (see below) high doses of the GAP sporozoites are used to infect mice in order to establish if these parasites completely arrest in the liver, an essential and critical safety requirement of a live-attenuated vaccine. In addition, mice immunized with GAP parasites (see below) are often challenged with relatively high doses of WT parasites (i.e. $1x10^4$), in

order to test the protective efficacy of different GAP vaccines and vaccination regiments. In sub-unit vaccine studies mice are usually challenged with lower doses of sporozoites (1-3 x 10^3), a dose reflective of 1-5 mosquito bites, after which parasite liver loads are established.

As well as transgenic RMP lines, reporter parasites have been generated for the human parasite *P. falciparum*. Transgenic *P. falciparum* parasites expressing fluorescent or luminescent proteins have been used to quantify blood stage growth *in vitro* in standard growth inhibition assays(see below),to quantify parasite development in the mosquito in standard membrane feeding assays to measure transmission-blocking (TB) activity and in high-throughput screening of TB compounds against *P. falciparum* gametocytes (see below). For the TB assays against mosquito stages and gametocytes, transgenic *P. falciparum* (NF54 strain)parasite lines have been generated that express a GFP-luciferase fusion protein under control of the strong constitutive *hsp70* [39] or the gametocyte specific *pfs16* promoter [40].

In addition to RMP expressing fluorescent and luminescent proteins for vaccine studies, multiple transgenic RMP lines expressing the model antigen OVA as an immunological reporter have been created to study immune responses after vaccination. Transgenic *Plasmodium* parasites expressing OVA have been exploited to examine parasite-specific $CD8^+$ T cell responses during both blood and liver infections [9, 10, 41-43]. For example, intravital two-photon microscopy of livers of mice infected with *P. berghei* parasites that express OVA and GFP in their cytoplasm showed that transferred OVA-specific $CD8^+$ T cells recognize and forms clusters around infected hepatocytes, leading to the elimination of the intra-hepatic parasites [41]. In addition, analysis of liver stage parasites expressing OVA, either in their cytoplasm or exported to the parasitophorous vacuole membrane, in conjunction with OVA-specific $CD8^+$ and $CD4^+$ OVA T cells demonstrated that export of parasite proteins into the infected hepatocytes enhanced immunogenicity and $CD8^+$ T cell based protection[10].

Below we describe the use of transgenic *Plasmodium* reporter parasites in preclinical assays to evaluate different *Plasmodium* vaccines and vaccination approaches, that target the 3 major points of the parasite life-cycle: erythrocytic vaccines, transmission blocking vaccines and pre-erythrocytic vaccines.

Erythrocytic vaccines

Although a number of RMP transgenic reporter parasites have been used in screening assays to evaluate drugs or other inhibitors, not many studies have reported the use of these parasites in assays to assess blood stage vaccines. The inhibitory activity of sera from (semi) immune individuals or purified immunoglobulins from vaccinated animals or people is mostly evaluated in *P. falciparum* using *in vitro* erythrocyte reinvasion and GIA assays. These assays are used to quantitatively measure antibody-mediated effects on parasite invasion and growth, often in small scale synchronized cultures of blood stage parasites that are maintained in microtiter plates for 1-2 cycles in the presence or absence

of antibodies. Determination of inhibition of invasion and growth in these assays is mainly performed by (automatic and high-throughput) microscopic, enzymatic or flow cytometric assays using wild type *P. falciparum* parasites [30, 44-46].In one study, a flow cytometric assay was developed that used transgenic *P. falciparum* parasites expressing GFP [16]. In this study *P. falciparum* parasites of the D10 strain were genetically modified to express GFP under control of the constitutive *Pfhsp86* promoter and inhibition of parasite growth by inhibitory antibodies and human serum was determined by measuring parasitemia by flow cytometry. This assay was superior to microscopy based approaches and comparable to DNA-staining based techniques to quantify growth inhibition (**Figure 1C**).

Transmission blocking vaccines

Mutant RMP are frequently used in (loss-of-function) studies that aim to identify and characterize *Plasmodium* proteins essential for parasite development in mosquitoes, which may be suitable targets for TB vaccine strategies. Often these deletion mutants have been created in transgenic RMP that express fluorescent or luminescent reporters, under control of constitutive stage specific promoters permitting a detailed examination of parasite development in the mosquito, for example enabling easier quantification of gametocyte development, fertilization and oocyst or sporozoite production. While the use of transgenic RMP in TB vaccine studies is limited, chimeric RMP lines expressing the ookinete surface protein P25 of *P. vivax* and *P. falciparum* have been used in direct mosquito feeding (DMF) assays for evaluation of the efficacy of vaccines targeting P25 of *P. vivax* and *P. falciparum*. In these assays immunized mice were challenged with the chimeric RMP parasites expressing the human antigen, followed by determination of oocyst reduction in mosquitoes that were fed on the immunized and challenged mice [44, 47-50]. The SMFA is a well-established and recognized method to evaluate TB activity of antibodies/serum against human malaria parasites [51]. This assay has been utilized widely to assess the TB activity of purified antibodies and serum, both in preclinical and clinical vaccine studies. TB activity in the SMFA is defined by the reduction in oocyst numbers in mosquitoes that have been fed with infected blood containing gametocytes in the presence of antibodies/serum compared to no(or control) antibodies(**Figure 1B**). Often oocyst production is measured by a microscopic analysis of dissected mosquito midguts. Recently, a transgenic reporter *P. falciparum* line expressing luciferase has been used in SMFA to quantify oocyst production in mosquitoes, thus eliminating the need for mosquito dissections[39]. This transgenic line was made in parasites of the *P. falciparum* NF54 strain and expresses a fusion protein of GFP and luciferase which is under control of the constitutive *Pfhsp70* promoter and parasites of this line do not express a drug-selectable marker. This novel dissection-free luminescence-based SMFA method, using a transgenic *P. falciparum* reporter parasite which is not resistance to known antimalarials, makes this assay much more amenable to high-throughput screening for both TB drugs and vaccines.

Pre-erythrocytic vaccines

Transgenic RMP are frequently used in preclinical sporozoite and liver stage vaccine studies. Simple and sensitive *in vitro* and *in vivo* assays employing luciferase-expressing *P. berghei* and *P. yoelii* parasites have been developed to visualize and quantify liver stage development [19, 22]. In these assays, parasite hepatic development is determined by bioluminescence measurement of cultured liver stages or by real-time imaging of luminescence emanating from the liver of live mice. These measurements correlate well with established (but more laborious) quantitative RT-PCR methods [38, 52]. Both *in vitro* and *in vivo* luminescence imaging assays have been used to screen inhibitors and vaccines against liver stages (**Figure 1C**; [23, 29, 31, 53, 54]).The simplicity and speed of quantitative analysis of parasite liver loads by real-time imaging and the possibility to analyze parasite development in live mice without surgery, greatly enhances the analysis of the effect of individual vaccines or vaccine strategies that target pre-erythrocytic stages. Quantification of parasite liver loads by real time imaging has been performed in mice that have been first vaccinated with human *Plasmodium* sub-unit vaccines and then challenged with luminescent chimeric RMP that express human parasite antigens (see **Section 3**) or in mice that have been immunized with genetically attenuated parasites and subsequently challenged with luminescent RMP(see **Section 4**). In addition, imaging of luminescent parasites in mice has been successfully used to examining host factors regulating liver infections[55]and to analyze the impact of immune responses on inhibition of liver stage development[23, 56-58]. Such studies have revealed the importance of adaptive and innate immune responses in protective immunity after vaccination. In these studies passively or actively immunized mice (including immunological compromised mice) were challenged with luciferase-expressing parasites to monitor reduction in parasite liver loads. In addition to the use of luminescent RMP, transgenic RMP expressing fluorescent proteins have been used to provide insight into interactions of sporozoites with cells in lymph nodes and with dermal tissue and blood vessels, and their interactions specifically with cells of the innate and adaptive immune system [59-64]. Using fluorescent *P. berghei* sporozoites it was demonstrated that fewer sporozoites enter the blood and reach the liver in sporozoite-immunized mice than naïve mice. Specifically, high circumsporozoite protein (CSP) antibody titers were shown affect sporozoite motility in the skin, preventing immobilized sporozoites of entering dermal blood vessels [65].

No assays have yet been reported to analyze *P. Falciparum* liver stage development *in vitro* with fluorescent or luminescent parasites. Most studies on *P. falciparum* liver stages, either cultured in hepatocytes (primary human or HC-O4 hepatocytes) or in chimeric mice with human liver tissue, have used wild type parasites that were analyzed by RT-PCR or by microscopy of fixed and stained cells. One study reported the use of transgenic *P. falciparum* parasites that express luciferase to study liver infection in immune compromised mice engrafted with human liver tissue [57]. This FRG huHep mouse is susceptible to a *P. falciparum* sporozoite infection and supports complete liver stage development. The reporter *P. falciparum* (NF54) parasites express a *gfp-luciferase* fusion gene under

the constitutive *Pfeef1a* promoter and the reporter expression cassette is introduced into the *pf47* locus [66]. In this study [57]a clear effect could be detected on infection of livers of FRG huHep mice by passively transferred antibodies against CSP and parasite liver loads in these mice were analyzed using bioluminescence imaging 6 days after infection with sporozoites (*i.e.*at the peak of liver-stage luciferase activity).

Chimeric rodent parasites expressing human plasmodium proteins

In addition to transgenic reporter parasites, rodent parasites expressing human malaria parasite proteins (HMP; *P. falciparum* and *P. vivax*) have been used in vaccine studies. These 'chimeric' RMP are used both to analyze immune responses against HMP antigens and to evaluate *in vivo* protective efficacy of vaccines that target HMP antigens (reviewed in [28, 29] and see **Table 1**). The preclinical evaluation of protective immunity involves mice being immunized with vaccines targeting different *P. falciparum or P. vivax* antigens followed by challenge with chimeric rodent parasites that express the corresponding HMP antigen. Mainly chimeric RMP expressing pre-erythrocytic HMP antigens have been generated (**Table 1**). Chimeric parasites have also been used to study immunogenicity and protective efficacy of transmission blocking HMP vaccine antigens, *i.e. P. falciparum* and *P. vivax* P25 [47, 49, 50]and blood stage vaccine antigens, *i.e. P. falciparum* MSP1 (**Table 1**).

Generation of chimeric parasites have been performed using standard methods of RMP transfection [67]by introducing HMP genes into the RMP genome, either as additional gene copies or by replacing the complete RMP with its HMP ortholog [29]. In addition, chimeric parasites have been generated that express fusions of the RMP and HMP orthologous genes (**Table 1**). The recently described GIMO (Gene Insertion-Marker Out) transfection method [68] greatly simplifies and speeds up the generation of transgenic parasites expressing heterologous proteins, which are free of drug-selection marker genes. Using this method two principle types of chimeric RMP expressing HMP proteins have been created ([29]; **Figure 2A**). The first type are 'additional copy mutants'; here the HMP gene is introduced as an additional gene copy into a silent/neutral locus of the GIMO mother-line and the HMP gene is under the control of a constitutive or stage-specific RMP gene promoter. This strategy is often used when an ortholog of the HMP gene is absent from the RMP genome. The second type of chimeric parasites are 'replacement mutants'; here the coding sequence (CDS) of the RMP gene is replaced with the CDS of the orthologous HMP gene. This method creates chimeric parasites expressing the HMP gene under control of the endogenous RMP gene promoter and transcriptional terminator. The absence of a drug-selectable marker in both the additional copy and replacement mutants makes it possible to rapidly introduce additional genetic modifications in these chimeric parasites, *e.g.* introduction of additional HMP genes or fluorescent/ luminescent reporter genes.

Chimeric parasites have been used in vaccine studies for a number of reasons. While a high level of genetic orthology exists between genes of RMP and HMP, critical differences

often exist in the sequence and structure of the encoded proteins [24]. In addition, HMP express a number of genes encoding vaccine candidates that are absent from RMP [24, 31].These differences complicate the analysis of immunogenicity and protective efficacy of HMP antigens in rodent models and compromise the effective translation of findings into a human malaria vaccine. Therefore 'humanizing' RMP by introducing HMP genes into rodent parasite genomes can help to circumvent some of these problems. HMP cannot readily infect small animals and testing of *P. falciparum* parasites in rodents is expensive as it is largely restricted to immune-deficient mice (*i.e.* DRAG or FRG) transplanted with human hematopoietic stem cells and/or liver tissue [69, 70]. While it is possible to test both pre-erythrocytic and blood stage *P. falciparum* vaccine candidates directly in human subjects, these studies are expensive and laborious to perform and therefore less suitable for larger screening studies[71]. Preclinical screening studies using chimeric RMP make it possible to rapidly evaluate and compare the protective efficacy of novel target antigens and vaccination strategies in order to down select candidate antigens and strategies that can proceed into clinical studies.

Recently 10 pre-erythrocytic *P. falciparum* vaccine candidate antigens were tested for their protective efficacy using chimeric parasites [31]. The antigens were selected based on published literature, immuno-profiling and expression studies. Mice, immunized with viral-vectored vaccines expressing the HMP antigens, were challenged with chimeric parasites for evaluation of protective immune responses and characterization of the immune responses (see **Figure 2B** for the immunization/challenge protocol). In this study two antigens, *Pf*LSA1 and *Pf*LSAP2, generated better protective efficacy than two leading pre-erythrocytic *P. falciparum* vaccine antigens, *Pf*CSP or *Pf*TRAP, in both inbred BALB/c and outbred CD-1 mice. The chimeric parasites used in this study had the HMP gene introduced as an additional gene copy as a number of the selected genes did not have an ortholog in the *P. berghei* genome, thereby excluding the possibility to make replacement mutants. A number of other chimeric RMP have been used, which express a HMP ortholog in place of their own RMP gene (**Table 1**), for example chimeric parasites expressing pre-erythrocytic vaccine candidates such as *P. vivax* and *P. falciparum* CSP and CelTOS ([72-75]; **Table 1**).

Chimeric parasites have also been used to evaluate immunogenicity of antigens against other lifecycle stages (*i.e.* TB vaccines see **Figure 2C**) as well as being used to evaluate different vaccine delivery platforms and to optimize the vaccination strategy and schedule. For example, the use of a single chimeric parasite expressing two HMP genes, TRAP and UIS3, showed that combination of two vaccines expressing these antigens could protect 100% of immunized mice, despite these antigens demonstrating only modest protective immunity when administered as a single antigen formulation [76]. This synergistic effect was only evident when the two vaccines were mixed and administered into two legs. Another study, testing different vaccine delivery platforms targeting *P. vivax* CSP using chimeric RMP that expressed *P. vivax* CSP, demonstrated that superior immunogenicity

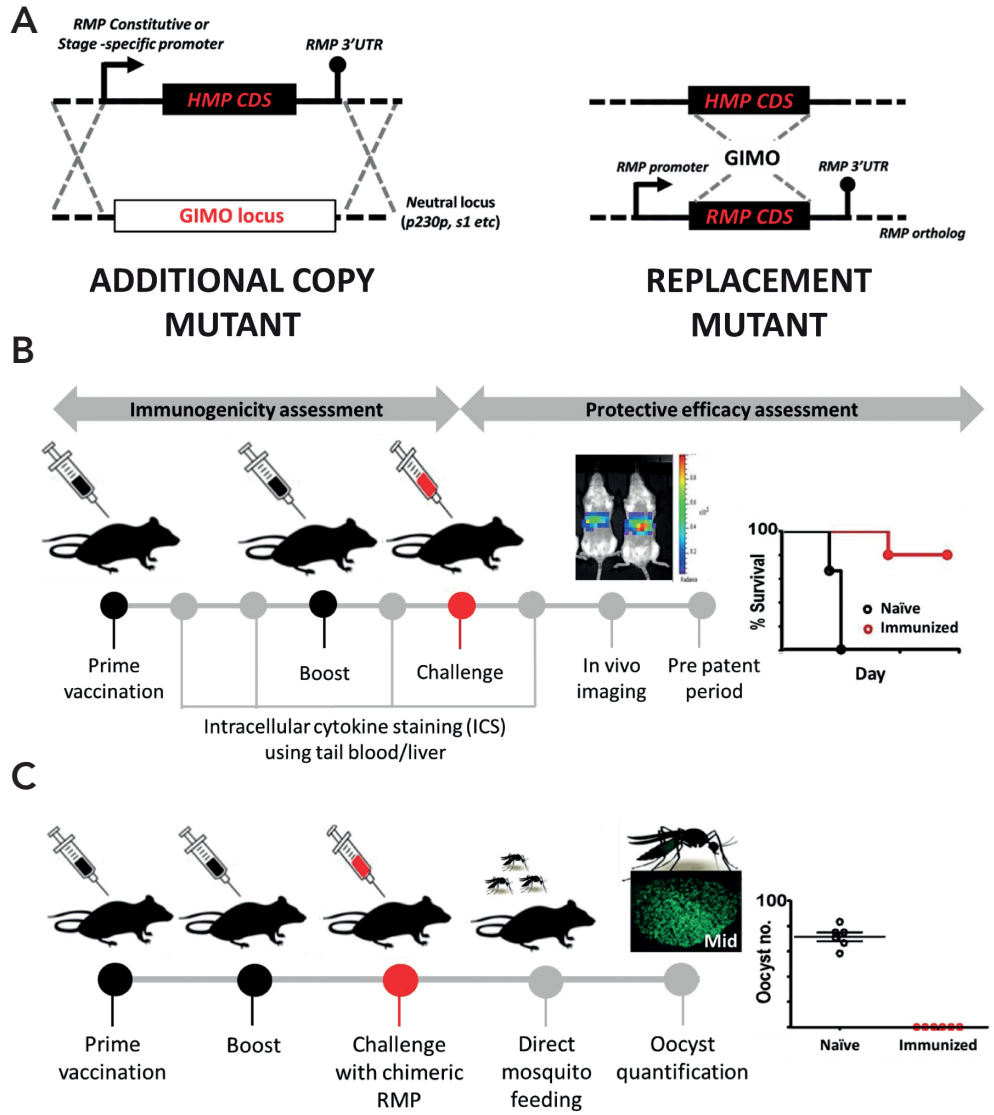

Figure 2. The use of chimeric RMP expressing human malaria parasite (HMP) proteins in malaria vaccine research. **A**. Additional Copy Mutants have the HMP gene (e.g. the *P. falciparum* gene coding sequence; *Pf CDS*) introduced as an additional gene copy into a silent/neutral locus of the RMP; the HMP gene is under the control of a constitutive or stage-specific RMP gene promoter. Replacement Mutants have the RMP coding sequence (*Pb CDS*) replaced by the orthologous HMP CDS. This often 2 step replacement method, employing the methods of GIMO transfection, creates chimeric parasites expressing the HMP gene under control of the endogenous RMP gene promoter and transcriptional terminator. **B.** Vaccine immunogenicity and protective efficacy measured in mice immunized with HMP liver stage sub-unit vaccines or rodent GAPs. Immunized (and naïve) mice are challenged either with luminescent chimeric RMP expressing the cognate HMP antigen or with luminescent 'wild-type' RMP. Protective efficacy, relative to unvaccinated mice, is quantified by measuring the parasite load by real time imaging of the liver of live mice at 44-48 h after challenge with sporozoites (*in vivo* imaging of luminescence) and/or measuring the time to establish a detectable blood stage infection

(pre-patent period; % survival). **C**. Vaccine efficacy of HMP transmission blocking vaccines determined in a direct mosquito feeding assay (DMFA) in mosquitoes. In these assays mice are immunized with the HMP transmission blocking vaccine. Immunized and naïve mice are then infected with chimeric RMP parasites expressing the cognate HMP antigen. The infected mice are used to feed mosquitoes and (reduction in) oocyst production in mosquitoes is quantified 8-10 days after feeding in order to measure of the transmission blocking potential of the HMP vaccine.

was generated by virus like particles (VLP) expressing *P. vivax* CSP compared to other formulations, including viral-vectored vaccines or protein plus adjuvant [77].

Chimeric parasites expressing either full length HMP proteins or fusions of HMP-RMP proteins can be instructive in determining critical immunological determinants of the protective immune responses after vaccination, for example in GIA using material obtained from immunized humans or animals [78-80]. However, the mechanisms of protection after vaccination can be lost in in vitro assays if only individual components of the adaptive immune response are examined in isolation. For example, responses that require both antibody and cell-mediated responses, either acting independently or when they work in concert such as in antibody-dependent cell-mediated cytotoxicity responses [81]. Ultimately, however, even positive results generated using chimeric parasites in rodents or in vitro assays will need to be validated in human vaccine trials.

Attenuated parasite vaccines

Transgenic parasites have not only been used for development and evaluation of immunogenicity of antigens and protective immunity of subunit vaccines, they have also been used to develop and evaluate whole organism vaccines consisting of (genetically) attenuated parasites. Vaccination with live, attenuated, sporozoites has been shown to induce strong protective immune responses both in rodents and in humans (reviewed in [32]). Sporozoite attenuation has been performed by radiation or by genetic modification of parasites (reviewed in [32-34, 82, 83]). A prerequisite for induction of protective immunity is that the attenuated sporozoites enter the liver, since heat-killed or over-irradiated sporozoites that do not invade hepatocytes do not efficiently confer protection [33, 84]. These so-called genetically attenuated parasites (GAPs) have genes encoding proteins essential for parasite development in the liver removed, thereby producing parasites that arrest in the liver. For both GAPs and radiation-attenuated parasites immunogenicity (protective efficacy) and safety are critical factors for further clinical development as whole organism vaccines. Transgenic rodent parasites have been used extensively in preclinical evaluation studies to establish the safety profile of GAPs, *i.e.* absence of a blood stage infection in mice after inoculation with high numbers of GAPs[34]. A number of different GAP vaccine candidates have been generated in rodent parasites, by deletion of either single or multiple genes. These have been analyzed in mice to ensure they completely arrest in the liver and therefore meet the necessary safety profile for translation into human GAP. Introducing genes encoding fluorescent and luminescent genes into the genomes of

GAPs has permitted a detailed analysis on the timing and magnitude of arrest in the liver [85, 86] (**Figure 1B**). Based on studies on growth arrest and safety of rodent GAPs, three multiple gene-deletion *P. falciparum* GAPs have been developed that have advanced into clinical evaluation [87-89].

In addition to examining the safety profile of a GAP, transgenic RMP have also been used to evaluate the protective immunity induced by attenuated sporozoites, both radiation-attenuated sporozoites and GAPs. In multiple studies, mice immunized with attenuated parasites have been challenged with fully infectious sporozoites that express luciferase to determine liver loads by real time imaging, similar to what has been described above for evaluation of protective immunity of sub-unit vaccines (**Section 2**and **3; Figure 2B**). Quantification of parasite liver loads and the pre-patent period provide a direct measurement of protective immunity induced by different immunization regimens.

Rodent GAPs expressing luciferase have also been used to investigate different attenuated sporozoite administration strategies [90, 91]. These studies demonstrated that the route and dose of administration of attenuated sporozoites are critical factors in inducing protective immunity. Intradermal, subcutaneous and intramuscular administration of attenuated sporozoites resulted in reduced parasite liver loads when compared to the same number of sporozoites introduced intravenously. Lower parasite liver loads after intradermal delivery was associated with reduced protective efficacy compared to intravenous immunization. Transgenic fluorescent rodent GAPs have been used to analyze direct interactions of lymphocytes with infected hepatocytes using intravital imaging of mice that had previously been immunized with attenuated sporozoites [13, 41, 92, 93]. These studies have revealed the importance of $CD8^+$ T cell mediated killing and elimination of infected hepatocytes in mice immunized with attenuated sporozoites. Further, using transgenic RMP expressing the immunological reporter protein ovalbumin, it has been possible to analyze direct interactions and effects of antigen specific $CD8^+$ T cell mediated immune responses in the liver of mice immunized with attenuated sporozoites ([10, 41]; see also **Section 2**).

Expert commentary

The ability to genetically manipulate the malaria parasite by deleting, mutating genes or introducing transgenes in the parasite genome has advanced our understanding of the molecular and cellular biology of malaria parasites for the last 20 years. Genetic modification has been central to the functional characterization of genes including genes encoding putative vaccine candidate antigens. The generation of reporter parasites with additional genes in their genome has resulted in the increased use of transgenic parasites in translation-oriented research, for example in preclinical studies evaluating immunogenicity and protective efficacy of novel antigens and vaccines. These studies involve transgenic parasites of both rodent and human malaria species. Two examples of transgenic human parasites are luminescent *P. falciparum* parasites that have been used in high-throughput

assays to quantify transmission blocking activity and the use of luminescent *P. falciparum* parasites to analyze the effects of (passively transferred) immune sera on liver infection in mice engrafted with human liver tissue (**Section 2**). These assays are used to generate insights into the immunogenicity of putative vaccine candidate antigens, knowledge which in turn can be used to improve vaccine strategies that target transmission blocking stages and pre-erythrocytic stages, respectively.

Compared to transgenic *P. falciparum* parasites, transgenic rodent malaria parasites have been more widely applied in experimental vaccine studies, especially in the evaluation of pre-erythrocytic antigens and to assess different pre-erythrocytic vaccination strategies. For example, luminescent parasites are frequently used to challenge immunized mice in standard assays that measure the reduction in parasite liver load as a consequence of the protective immune responses induced by different antigens or vaccine strategies. Another example is the application of intravital imaging using fluorescent parasites in immunized mice, which has revealed critical insights into the immune response targeting sporozoites and infected liver cells (**Section 2**). Such *in vivo* assays to analyze crucial protective immune responses after vaccination and to evaluate protective immunity are valuable tools to improve pre-erythrocytic vaccines.

In addition to reporter rodent parasites, chimeric rodent parasites expressing proteins of the human malaria parasites *P. falciparum* and *P. vivax* are now being increasingly used in vaccine studies. Chimeric RMP expressing HMP proteins are used to determine protective efficacy in mice immunized with different sub-unit vaccines expressing *P. falciparum and P. vivax* antigens (**Section 3**). These studies have been used to select novel vaccine candidate antigens for advancement into clinical trials. Chimeric RMP can not only support identification of novel antigens, but also contribute to the *in vivo* evaluation of novel delivery platforms and vaccine strategies, both for vaccines targeting pre-erythrocytic parasites and transmission blocking vaccines (**Section 3**). The use of chimeric rodent parasites to evaluate protective immunity or transmission blocking immunity is not without its limitations. First, the use of chimeric RMP still relies on a murine model, often inbred mice strains, and encounter issues related to restriction of MHC epitopes and marked immune-dominance of certain epitopes [94]. Outbred mice can possibly be used to more accurately reflect what may be seen in humans but it is possible that some antigens identified as poorly immunogenic in these studies may in fact be immunogenic in humans. Second, when using 'Additional copy' chimeric parasites, the HMP gene expression is dependent on the RMP promoter used, which is unlikely to exactly mimic the timing and magnitude of the expression of the HMP protein in the HMP. In studies where multiple vaccine antigens are examined the chimeric parasites will express the different HMP antigens at the same level, which is unlikely to be the case in wild-type HMP. Therefore, where possible, it would be useful to also compare protective vaccine efficacy in mice using a chimeric RMP parasite where the HMP antigen expression matches its expression in the HMP, both in timing and magnitude. Despite these limitations, chimeric RMP allow for rapid vaccine (rank-order) screening *in vivo* and can provide critical insights into both

the importance of the vaccine target and the mechanism of protection. Indeed data from chimeric RMP is being used to justify the selection of novel HMP antigen vaccines (and delivery platforms) to advance into clinical testing.

In addition to the role of transgenic parasites in the development of subunit vaccines, transgenic parasites have played a central role in the development and evaluation of whole organism vaccines consisting of attenuated sporozoites. Studies in rodent malaria models on the safety and immunogenicity of GAPs has formed the basis of the development of different (multiple gene deletion) *P. falciparum* GAPs that have now advanced into clinical trials (see **Section 4**). Given the data from rodents studies with both GAPs and irradiated sporozoites and from data emerging from irradiated sporozoite vaccine research in humans it is anticipated that further improvements can be made to increase GAP potency. Here again transgenic RMP can play an important role, for example to optimize the routes of attenuated parasite vaccine administration (e.g. studies with devices to improve intradermal or intramuscular delivery, use of adjuvants *etc*) and in development of the so-called 'next generation' GAP vaccines with increased potency requiring fewer sporozoites per dose and fewer vaccination doses to achieve sustained sterile protection (e.g. GAPs which arrest late into liver stage development).

Transgenic parasites used in conjunction with 'humanized' animal models or in sophisticated in vitro assays are designed to aid and speed up malaria vaccine design, specifically to suggest potential priorities for expensive and time-consuming clinical trials. As mentioned above, however, the predictive power of these assays can only be determined after human trials have been performed and lessons learnt from the success and discrepancies that will arise. In addition, over-reliance on a single experimental model may result in putative valid vaccine targets not being advanced further, as they did not generate sufficient immunity in the testing platform (e.g. in mice).

Five-year view

Despite considerable effort, over decades, a highly effective vaccine against malaria still does not exist. This is in part due to the limited number of antigens and methods of immunization that have advanced into clinical testing. Most vaccine studies have focused on a limited number of antigens but for a broad acting, highly durable and potent malaria vaccine this is likely to be too restrictive and insufficient to provide the protection required. Therefore, in order to create multi-antigen and multi-stage vaccines many more antigens and improved vaccine delivery platforms will need to be investigated and evaluated as a priority in the next 5 years. In addition, the critical host and parasite factors mediating protective immunity and those that are necessary for maintaining durable protection need also further investigation in the upcoming years. The use of transgenic parasites in conjunction with other enabling technologies (e.g. genetic modification of mice or human cell lines, advances in imaging *etc*) has opened up new possibilities and will be used to contribute to a more rapid preclinical evaluation of vaccines, vaccination strategies and

identification of critical factors of protective immune responses. Transgenic *P. falciparum* parasites expressing luminescent reporter proteins are currently valuable tools to assess drugs and inhibitors against the parasite in high-throughput assays and are now also being used to test the immunogenicity of (novel) transmission blocking antigens and will continue to be used to evaluate novel transmission blocking vaccine strategies. In addition, the recent availability of luminescent *P. falciparum* parasites that express luciferase under strong promoters (i.e. constitutive, sporozoite or liver-stage specific) will act as a bridge between rodent and clinical studies. They will be increasingly used in assays to evaluate the effects of (human) immune serum, cells and factors on *P. falciparum* blood and liver cell infection, both in cultured cells and in humanized mice with human hematopoietic and human liver cells. Such assays will contribute to generate essential insights into the immunogenicity of (in particular pre-erythrocytic) antigens and vaccination strategies. Both reporter RMP expressing fluorescent and luminescent proteins as well as chimeric RMP expressing HMP antigens will contribute to these studies examining protective immune responses in particular of vaccine strategies targeting pre-erythrocytic vaccines. The use of transgenic parasites may not only help to rank order existing candidates but also help to reveal novel vaccine candidate antigens and vaccination strategies. Loss of function and protein-tagging mutants often reveal parasite proteins that have critical roles in parasite development or, for example, are located on the surface of extracellular forms of the parasite and may therefore be vulnerable to antibody-based vaccines. Uncovering critical protective immune responses and efforts to establish correlates of protection after vaccination may be greatly aided by the use of both transgenic parasites and humanized mice, which could be used to examine both the induction and recall of immune responses in different organs. Transgenic RMP will continue to play an important role in preclinical evaluation of novel attenuated sporozoite vaccines both in studies to develop GAPs that are more immunogenic and in studies to improve vaccination strategies (e.g. optimizing the route of administration). In particular, next generation *P. falciparum* GAPs that have been further modified to express multi-stage and antigens from multiple strains.

Key issues

- Most vaccine studies have focused on a limited number of antigens but for a broad acting, highly durable and potent malaria vaccine this is likely to be too restrictive and insufficient to provide the protection required. Multi-stage, multiple-antigen sub-unit or genetically attenuated parasite vaccines may provide a solution.
- Transgenic (human and rodent) malaria parasites expressing 'foreign' proteins, for example fluorescent and luminescent proteins, have been used to determine the protective efficacy of different antigens and to evaluate vaccination platforms/strategies.
- Transgenic parasites (e.g. expressing OVA) are being used to understand the critical determinants of protection after vaccination; specifically to examine the induction and recall of protective immune responses in the blood and the liver

- Luminescent rodent parasites are now increasingly used to challenge vaccinated mice, and non-invasive measurements of parasite liver load permits examination of both the protective responses generated by different antigens and to evaluate novel vaccine strategies.
- Luminescent *P. falciparum* parasites are being used both in high-throughput assays to quantify transmission blocking activity and to analyze the effects of human immune sera/immunoglobulins on parasite development in the liver of humanized mice.
- Chimeric rodent parasites, expressing *P. falciparum* or *P. vivax* antigens, are being used to directly evaluate and rank-order human malaria vaccine candidates and determination of the most suitable for clinical testing.
- Chimeric rodent parasites permit an *in vivo* comparison of different *P. falciparum/ vivax* vaccine delivery platforms and vaccination strategies; they are being used to determine the best combination of antigens, delivery system and immunization protocol to move forward into clinical testing.
- Transgenic parasites play a central role in the development and evaluation of whole organism vaccines consisting of attenuated sporozoites. Both in evaluation of safety and in assessing protective efficacy. Improvements in genetically attenuated parasite vaccines and strategies for vaccination (i.e. optimizing the route of administration) will continue to require the use of transgenic parasites.

Ackowledgements

A.S Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). C Marin-Mogollon is supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). A. M Salman is supported by Prof. Adrian Hill's Senior Investigator Award from the Wellcome Trust (095540/Z/11/Z). S Khan, B.M Franke-Fayard and C.J Janse are full time employees of the Leiden University Medical Center (LUMC).

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Reference annotations

* Of interest

** Of considerable interest

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CHAPTER 3

Rapid generation of marker-free *P. falciparum* fluorescent reporter lines using modified CRISPR/cas9 constructs and selection protocol

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PLoS One. 2016 Dec 20;11(12):e0168362

Abstract

The CRISPR/Cas9 system is a powerful genome editing technique employed in a wide variety of organisms including recently the human malaria parasite, *P. falciparum*. Here we report on further improvements to the CRISPR/Cas9 transfection constructs and selection protocol to more rapidly modify the *P. falciparum* genome and to introduce transgenes into the parasite genome without the inclusion of drug-selectable marker genes. This method was used to stably integrate the gene encoding GFP into the *P. falciparum* genome under the control of promoters of three different *Plasmodium* genes (*calmodulin, gapdh* and *hsp70*). These genes were selected as they are highly transcribed in blood stages. We show that the three reporter parasite lines generated in this study (GFP@*cam*, GFP@*gapdh* and GFP@*hsp70*) have *in vitro* blood stage growth kinetics and drug-sensitivity profiles comparable to the parental *P. falciparum* (NF54) wild-type line. Both asexual and sexual blood stages of the three reporter lines expressed GFP-fluorescence with GFP@*hsp70* having the highest fluorescent intensity in schizont stages as shown by flow cytometry analysis of GFP-fluorescence intensity. The improved CRISPR/Cas9 constructs/protocol will aid in the rapid generation of transgenic and modified *P. falciparum* parasites, including those expressing different reporters proteins under different (stage specific) promoters.

Introduction

A wide variety of transgenic parasite lines have been generated in rodent malaria parasites, including those that express fluorescent and/or luminescent reporter proteins under the control of constitutive or stage-specific promoters. Such transgenic 'reporter' parasites have proven to be useful tools to interrogate *Plasmodium* gene function, examine the effect of inhibitors on parasite development, to evaluate sub-unit vaccine efficacy *in vivo* and to rank order and evaluate live-attenuated parasite vaccines [1-12]. For rodent malaria parasites technologies have been developed to stably introduce transgenes into the parasite genome and efficient and rapid methods exist for the generation of reporter parasite lines that do not contain drug-selectable markers [13, 14]. Such 'marker-free' parasites make it considerably easier to further genetically modify transgenic parasites and, moreover, they can be used for drug-sensitivity testing, as possible interference from an introduced drug-selection marker is absent. In rodent malaria parasites such reporter parasite lines have been generated in multiple strains of three different *Plasmodium* species [15].

In comparison to rodent malaria parasites the technologies to genetically modify the human malaria parasite, *P. falciparum*, are much less efficient [16] and the number of stable reporter parasite lines in different *P. falciparum* strains is limited [17, 18]. In addition, currently no cloned reporter lines have been published that are drug-selectable marker free. The traditional approaches to engineer the *P. falciparum* genome have been hampered by the limited methods available and transfection inefficiencies in introducing exogenous DNA into the parasite genome. Also the limited number of drug-selectable markers restricts genetic engineering of *P. falciparum*, for example, performing sequential genetic manipulations in the same parasite line. Several technologies have been developed for the removal (re-cycling) of drug-selectable markers from the modified parasite genome, specifically using either FLP or Cre recombinases [19, 20]. However, the application of these techniques are time consuming as it can take 4-5 months to generate cloned 'marker-free' genetically modified parasites.

The RNA-guided CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system has transformed genome editing in a wide variety of organisms [21]. This powerful genome editing technique has also been applied to *P. falciparum* and provides an efficient method to manipulate the parasite's genome, such as site directed mutagenesis, gene disruption and the introduction of transgenes [22, 23]. The CRISPR/Cas9 method is based on the initial generation of site-specific double strand DNA break induced by a Cas9 endonuclease and subsequent repair and modification of the DNA locus. The Cas9 enzyme is guided to a specific site in the genome by a single guide RNA (sgRNA) that can be modified to specify the exact DNA sequence within the genome. The presence of a template or 'donor DNA' that contains sequences surrounding the double stranded DNA break can result in guided (or homology directed) repair, resulting of introduction of donor DNA at the site of the break [24]. Frequently

a two plasmid approach is used to introduce Cas9, the single guide RNA (sgRNA) composed of a fusion between CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA), and donor DNA into the nucleus of the organism. *P. falciparum* transfections have been performed with Cas9 and sgRNA either expressed on two separate plasmids or combined on one plasmid and different selectable markers have been used to maintain the plasmids in transformed parasites after transfection [22, 23, 25-27]. The selectable markers used are human dihydrofolate reductase (h*dhfr*), blasticidin S deaminase (*bsd*), neomycin phosphotransferase (*neo*) and yeast cytosine deaminase/uridyl phosphoribosyl transferase (y*fcu*). Generation of *P. falciparum* transgenic reporter parasites would benefit from the availability of standard CRISPR/Cas9 plasmids that permit the rapid introduction of different transgenes into the parasite genome without permanently integrating a drug-selectable marker cassette. Recently improved CRISPR/Cas9 constructs have been reported for marker-free editing of the *P. falciparum* genome [26]. One plasmid contains Cas9, the sgRNA and a *bsd* selectable marker cassette, whereas the other construct, containing the donor DNA, does not encode a drug selectable marker. The use of this 'marker-free' construct thus can permit an introduction of larger donor DNA sequences. Using these constructs marker-free GFP-expressing parasites have been reported.

In this paper we describe the generation of marker-free reporter parasites by using modified CRISPR/Cas9 constructs compared to the constructs described in previous studies. We generated a standard plasmid that encodes Cas9 and contains the *bsd* selection marker cassette. The sgRNA and donor DNA are both present on a second plasmid, which contains the positive selectable marker, h*dhfr*, fused to the negative selectable marker, y*fcu*. This dual positive-negative selectable marker cassette is not integrated into the genome but is used to rapidly select 'marker-free' transgenic parasites by the successive application of positive drug selection followed by negative selection. By generating three reporter lines which stably express GFP under the control of different promoters we show that cloned marker-free reporter parasite lines can be obtained within a period of 10-12 weeks. In addition, we show that these reporter parasites have the same *in vitro* blood-stage growth kinetics and drug-sensitivity profiles as the parental wild-type parasites and we compared the relative strengths of the different promoters to drive GFP expression. The constructs and selection protocol described in this study provide a simple set of tools to rapidly generate modified *P. falciparum* lines, in particular transgenic parasites that can be used to examine different *Plasmodium* regulatory elements to control transgene expression. The same constructs can be used to perform other genetic modifications, for example gene disruption or gene mutation, to interrogate gene function and can be used to perform rapid and multiple successive genetic manipulations.

Results

Improved CRISPR/Cas9 plasmids to introduce transgenes into the Plasmodium genome without the addition of drug selectable markers

Homologous recombination using the CRISPR/Cas9 protocol requires introduction of the Cas9 endonuclease complexes with a sgRNA and DNA sequences (donor DNA) that will induce a double stranded break in the genome and then repair the target region. Often these different elements are present on two different plasmids encoding different drug-selectable markers. To introduce Cas9 we used the plasmid described by Ghorbal *et al.* [22]. However, in this construct (pLf0019) we replaced the y*dhodh* drug-selectable marker (SM) by the more standardly used *bsd* SM (**Figure 1**), as the drug Blasticidin (BSD) is easier to obtain than DSM1 that is used in conjunction with the y*dhodh* SM.

A second plasmid (pLf0022) was generated that both contains the donor DNA, the sgRNA expression cassette and drug-selectable marker. For sgRNA we used the expression cassette of the published plasmid pL6-eGFP [22], which contains the BtgZ1 adaptor sequence and the tracrRNA sequence under control of the *Plasmodium u6* RNA promoter. The drug-selectable marker cassette we used is a fusion of the positive selectable marker h*dhfr* (human dihydrofolate reductase), and the negative selectable marker y*fcu* (*yeast cytosine deaminase/uridyl phosphoribosyl transferase*) [28]. This fusion gene, h*dhfr::*y*fcu*, was placed under control of the *P. falciparum hsp86* promoter and the *P. berghei dhfr/ts* transcriptional terminator (3'UTR) and the positive-negative drug selection marker was tested in a transient transfection drug-sensitivity assay. In this assay, parasites of the *P. falciparum* NF54 line were transiently transfected with a circular plasmid (pLf0033; **Figure S1A**) encoding the h*dhfr::*y*fc* fusion protein and were treated with either WR99210 (positive) or 5-FC (negative) for 12-16 days. The transiently transfected parasites treated with WR99210 exhibit a growth rate comparable to untreated NF54 wild type parasites (**Figure S1B**), whereas treatment with negative drug selection (using 5-FC) killed transfected parasites (**Figure S1C**). The h*dhfr::*y*fcu* fusion cassette therefore confers both resistance to WR99210 and sensitivity to 5-FC and can be used efficiently for positive and negative selection in *P. falciparum* transfections. We reasoned that by applying first positive selection (with BSD and WR92210) followed by negative selection (with 5-Fluorocytosine, 5-FC), would improve the selection of parasites where the donor DNA had been integrated into the genome. Specifically, applying first positive selection will select for parasites that contained both plasmids, resulting in a DNA break followed by donor DNA mediated repair of the target locus. Once parasites were visible in blood stage cultures we applied negative selection to select only parasites free of episomal plasmid DNA. Moreover, we placed the selectable marker fusion cassette outside the donor DNA cassette in the plasmid (**Figure 1**). This location permits the introduction of donor DNA sequences into the target locus without the introduction of a drug-selectable marker into the parasite genome (**Figure 1**). The fusion of the positive and negative selectable marker cassette also reduces the size of the overall plasmid compared to a construct where both

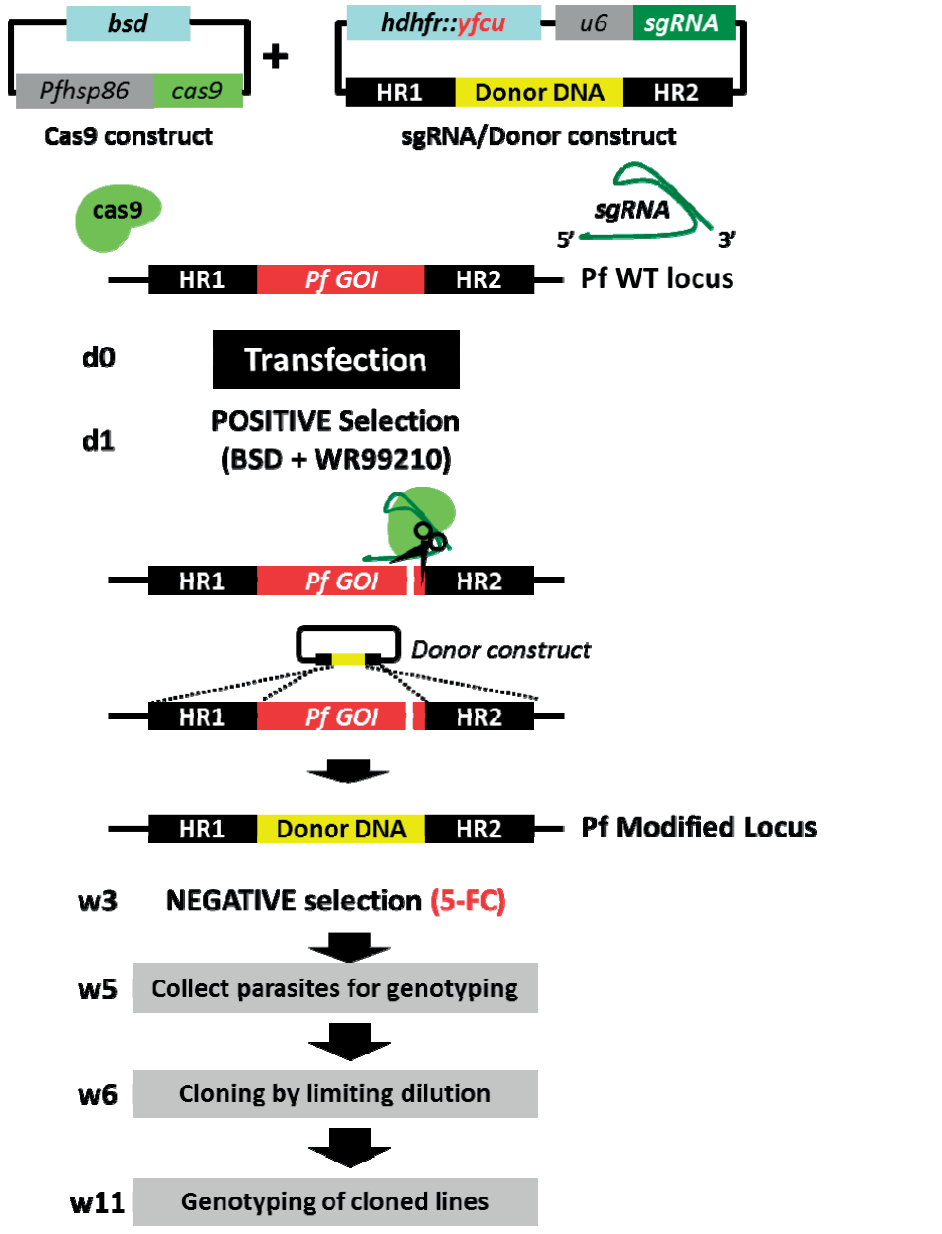


Figure 1. Schematic representation of improved CRISPR/Cas9 plasmids and selection protocol. Parasites are transfected with two plasmids (Cas9 construct and sgRNA/donor construct). The Cas9 construct contains the *bsd* selectable marker. The sgRNA/donor construct contains a fusion of the positive selectable marker h*dhfr* and the negative selectable marker y*fcu* genes and two homology regions (HR1 and HR2) that target a gene of interest (GOI) and introduce the donor DNA by homologous recombination. Double positive selection using both BSD and WR99210 is applied from day (d) 1 resulting in the selection of parasites that contain both plasmids within a period of 3 weeks (w). After positive selection, cultures are maintained 2-4 days without drug before negative selection is applied using 5-FC to select parasites free of episomal plasmid DNA. Parasites are genotyped by diagnostic PCR for integration of the donor DNA followed by cloning of the parasites by limiting dilution (w6). Clones are genotyped for the correct genotype by diagnostic PCR and Southern analysis. This transfection and selection protocol can result in the generation of cloned mutant parasites within a period of 11 weeks.

selectable markers were controlled by separate regulatory elements. This increases the size of heterologous DNA that can be introduced as donor DNA; in **Figure S2** the plasmid maps of both the Cas9 (pLf0019) and the crRNA/Donor (pLf0022) are shown in more detail.

Generation of (donor DNA) constructs to introduce different GFP-expression cassettes into the P. falciparum genome

To introduce transgenes into the *P. falciparum* genome we further modified pLf0022, and introduced homology sequences to target the *P. falciparum p230p* (*Pf230p*) gene locus. The *Pf230p* gene is not transcribed in asexual blood stages [29] and is therefore unlikely to be essential for asexual blood stage development/multiplication. Homology region 1 (HR1) and homology region 2 (HR2) were both PCR amplified from *P. falciparum* (NF54) genomic DNA and cloned into plasmid pLf0022.

A 20 nucleotide crRNA sequence specific for *Pf230p* was identified using Protospacer software and this crRNA was introduced into the pLf0022 by replacing the *Btg*ZI adaptor sequence, resulting in a *Pf230p* sgRNA (sgRNA2) in the construct. This modified pLf0022 vector containing two HR of *Pf230p* and sgRNA2 created the plasmid pLf0024. We identified promoters to drive strong GFP transgene expression in blood stages. Specifically, promoters of three strong constitutively expressed genes based on published transcriptional profiles (RNAseq) data available from the PlasmoDB database (www.plasmodb.org). Genes were selected that had transcript levels (RNAseq values) that were similar or higher than that of the constitutively expressed *elongation factor 1α* (PF3D7_1357100), the promoter of this gene has been previously used in both *P. falciparum* and rodent models of malaria to drive the expression of reporter proteins [30]. These promoters were from the following genes: *calmodulin* (PF3D7_1434200; *cam*), *glyceraldehyde-3-phosphate dehydrogenase* (PF3D7_1462800; *gapdh*) and *heat shock protein 70* (PF3D7_0818900; *hsp70*); see **Table S2** for data on the transcript levels of these genes. In the *cam* and *gapdh* promoter GFP-expression cassettes the *gfp* gene was placed under control of the *P. berghei calmodulin* transcriptional terminator (3' UTR), whereas for *hsp70* the 3'UTR of *P. falciparum histidine-rich protein II* was used. In the final constructs the GFP expression cassette of *gfp@cam* and *gfp@gapdh* are consequently in a different orientation to *gfp@hsp70*; see the Material and Methods section for further details. Cloning of the different GFP expression cassettes in pLf0024 resulted in the following constructs; *gfp@cam* (pLf0026), *gfp@gapdh* (pLf0032) and *gfp@hsp70* (pLf0035), (**Figure 2A and S2**).

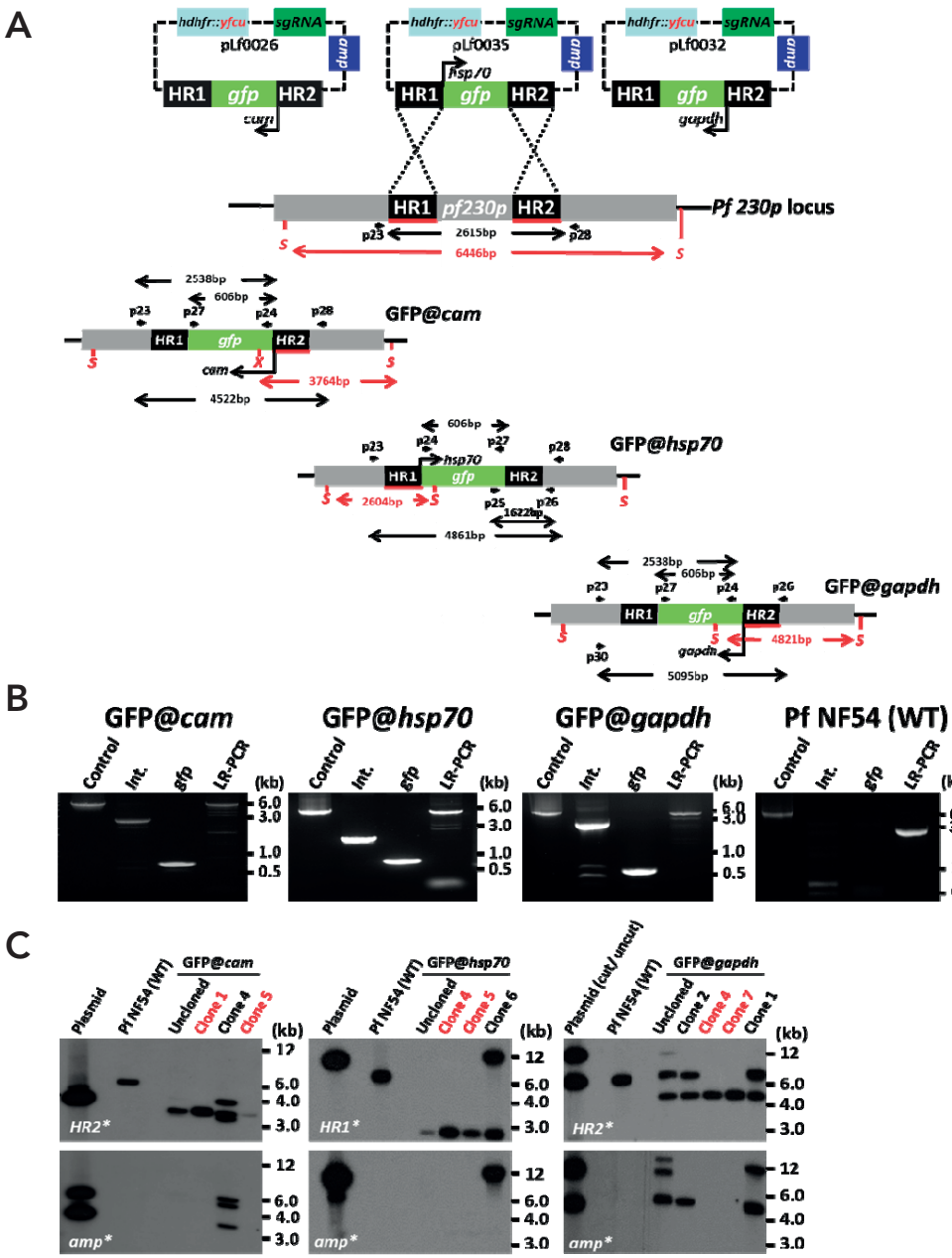


Figure 2. Generation of three *P. falciparum* reporter lines (GFP@*cam*, GFP@*hsp70*, GFP@*gapdh*) expressing GFP under control of different promoters. **A.** Schematic of the different sgRNA/donor constructs generated to introduce the GFP expression cassettes into the *P. falciparum* (*Pf*) *230p* gene locus. *Pf230p* homology regions (HR1, HR2) used to introduce the donor DNA (i.e. *gfp* expression cassettes), location of primers (p) and sizes of restriction fragments (S: *Spe*I, X: *Xho*I; in red) and PCR amplicons (in black) are indicated. Primer sequences (shown in black and bold) are shown in **Table S1**. Note that the GFP expression cassette from GFP@*cam* and GFP@*gapdh* was cloned in the same orientation whereas that the GFP expression cassette form GFP@*hsp70* was cloned in the reverse orientation. See **Figure 1 and S1** for details of the drug selectable marker and sgRNA sequences. This Figure is not shown to scale. **B.** Diagnostic (first 3 lanes) and long-range (LR-) PCR confirming correct integration of the GFP-expression cassettes into the *Pf230p* locus. Integration PCR of cloned parasites of GFP@*cam* (clone 1; primers p23/p24; expected size: 2538bp), GFP@*hsp70* (clone 5; primers p25/p26; expected size: 1622bp) and GFP@*gapdh* (clone 7; primers p23/p24; expected size: 2538bp). LR-PCR: GFP@*cam* (primers p23/p28; expected size: 4522bp), GFP@*hsp70* (primers p23/28; expected size: 4861bp) and GFP@*gapdh* (primers p30/p26; expected size: 5095bp); size of expected products shown in black and in bold in Figure 2A. Control PCR: *P. falciparum lisp2* gene (primers p21/p22; expected size: 5383bp); GFP: *gfp* gene (primers p24/p27; expected size: 606bp). **C**. Diagnostic Southern analysis confirms correct integration of the GFP-expression cassettes in the cloned lines of GFP@*cam*, GFP@*hsp70* and GFP@*gapdh*. *P. falciparum* NF54 (wild type WT) DNA, transfected parasite DNA after positive and negative selection (Uncloned; see **Figure 1**) and DNA from the different cloned lines was digested with *Spe*I and/or *Xho*I. The digested DNA fragments hybridized to probes recognizing either HR1 (GFP@*hsp70;* expected size: 2604bp) or HR2 (GFP@*cam;* expected size: 3764bp and GFP@*gapdh;* expected size: 4821bp) of the *Pf230p* target locus. In red are indicated the clones that have the correct genotype; absence of both plasmid and WT DNA (clone 1 and 5 for *GFP@cam;* clone 4 and 5 for *GFP@hsp70;* and clone 4 and 7 for *GFP@gapdh).* As controls sgRNA/donor plasmid (Plasmid) DNA was digested and hybridised with a probe recognizing *ampicillin* (amp) of the donor DNA plasmid; *indicates probe used.

Generation of three transgenic reporter P. falciparum lines expressing GFP under different promoters

All construct were used to transfect *P. falciparum* ring stage parasites that were obtained from cultures after sorbitol synchronization. In each transfection 300 µl of pelleted infected RBC from cultures with a 6-15% parasitemia were mixed with 50 µg of the Cas9 and 50 µg of the donor plasmid. After transfection parasites were cultured in 10 ml flasks of an semi-automated culture system [31]. Twenty-four hours after transfection we applied 'double' positive selection by adding the drugs WR99210 and BSD to the cultures to select only for parasites that contain both plasmids. Drug pressure was maintained until infected RBC were detected by thin blood-smears analysis (usually 3 weeks after transfection). Subsequently, both drugs were removed from the cultures for 2-4 days, followed by the application of negative drug selection by the addition of 5-FC. This was performed in order to remove parasites that still retain episomal donor construct plasmid DNA, thereby enriching for transfected parasites that have the donor DNA integrated into their genome. To avoid any potential bystander killing effect of 5-FC at higher parasitemias we treated the cultures with 5-FC only after reducing the culture parasitemia to 0.5%. Negative drug pressure was maintained until thin blood-smears were parasite-positive (usually 7 days after application of 5-FC; **Figure 1**).

During both the positive and negative drug selection, parasites were analysed for GFP expression by fluorescence microscopy to determine the ratio of wild type and transgenic parasites present in the population. In multiple transfection experiments (exp.) with the three constructs with the different GFP-expression cassettes we obtained GFP-positive parasites after positive and negative selection (exp. 22 and 33 for pLf0026; exp. 44 for pLf0032 and exp. 35 for pLf0035). After negative selection the ratio of GFP+/GFP- parasites

was determined and in the positive experiments the percentage of GFP-positive parasites ranged between 70 and 90%. Diagnostic PCR analysis for double cross-over integration after negative selection confirmed the presence of parasites with correct integration of the donor DNA. Based on the high GFP+/GFP- ratios and positive diagnostic PCR we proceeded to clone parasites from the following transfections exp. 22 (GFP@*cam*), exp. 35 (GFP@*hsp70*) and exp. 44 (GFP@*gapdh*). Cloning was performed by limiting dilution and GFP-positive clones were cultured in the semi-automated culture system for further genotyping by diagnostic PCR and Southern analysis. Both analyses confirmed correct integration of the donor DNA into the genome of the three cloned transgenic lines GFP@*cam* (exp. 22 clone 1 and 6), GFP@*hsp70* (exp. 35 clone 4 and 5) and GFP@*gapdh* (exp. 44 clone 4 and 7) and absence of the wild type DNA (**Figure 2B,C**). The cloning experiments indicated that >50% of the cloned lines we generated had the desired integration (i.e. GFP@*cam* 66%, 3 clones analysed; GFP@*gapdh* 57%, 7 clones analysed; and GFP@*hsp70* 66%, 3 clones analysed) (**Figure 2C**). In these 3 independent transfections the time from transfection to obtaining the marker-free GFP-expressing clones ranged between 10 and 12 weeks.

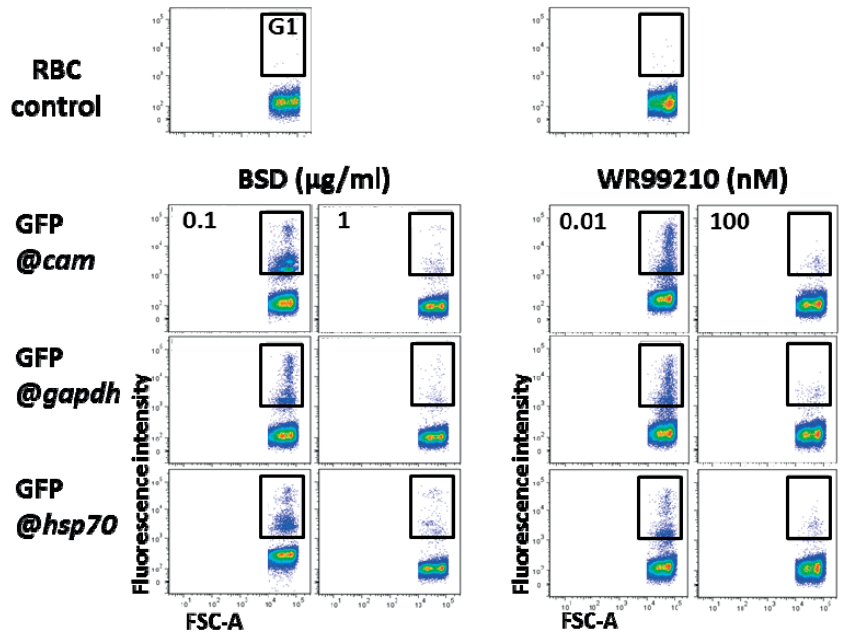
P. falciparum reporter lines retain WT-like growth kinetics and drug sensitivity during blood stage development

The three reporter lines are free of a drug-selectable marker and consequently are easier to further genetically modify. In these reporter lines, using the same constructs described above, it is possible to delete, mutate or tag *P. falciparum* proteins in order to investigate their function and importance during parasite development. A prerequisite for additional genetic modifications using DNA constructs described above is that the parasite retains the same sensitivity to the drugs used to select parasite after transfection. It has been reported that parasites can spontaneously acquire blasticidin resistance when exposed to sustained BSD treatment independent of the *bsd* selectable marker [32]. We therefore compared the sensitivity of the three parasite lines to BSD and WR99210.

The drug-sensitivity of the asexual blood stages of clones of the three transgenic lines was determined in standard 72 h short-term culture assays in 96-wells culture plates. Serial dilutions of BSD and WR99210 were made with concentrations ranging from 0.1 to 1 µg/ml or 0.01 to 100 nM, respectively. Parasitemias in the culture wells were determined by flow cytometry and the parasite survival rate calculated (**Figure 3A**). The drug-sensitivity curves of the three reporter lines are comparable to that of wildtype NF54 parasites, with IC50 values between 0.34 and 0.54 µg/ml for BSD and 0.16 and 0.27 nM for WR99210 (**Figure 3A**).

In addition, in order to use these reporter lines to analyse the effect of additional modifications and/or inhibitors on the growth characteristics of *P. falciparum* blood stages, it is important that these parasites retain growth and development kinetics of the parental NF54 strain. We therefore compared the growth rate of the three different reporter lines. The growth rate of asexual blood stages was monitored by flow cytometry of samples

A



B

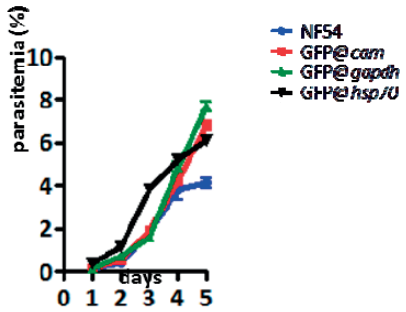


Figure 3. Drug-sensitivity and growth rate of asexual blood stages of three *P. falciparum* reporter lines (GFP@*cam*, GFP@*gapdh*, GFP@*hsp70*)**. A**. Sensitivity to the drugs BSD and WR99210 as determined by flow cytometry in standard 72 h cultures in 96 well plates. Cultures of infected red blood cells (RBC) were incubated with different drug concentrations (in triplicate) and after 72 h samples were stained with the DNA-specific dye, Hoechst 33258, to determine parasitemia (% of infected RBC) by flow cytometry. Dot plots are shown of uninfected RBC (control, upper panel) selected using

Forward Scatter parameter (FSC-A) and from cultures with the lowest and highest drug concentration (G1: infected RBC). Parasite survival is defined as the percentage of infected RBC in drug-treated wells divided by the percentage of infected RBC in non-treated wells multiplied by 100. IC_{50} values WR99210 (nM): (NF54 *Pf*WT) 0.16; (GFP@*cam*) 0.25; (GFP@*gapdh*) 0.27; (GFP@*hsp70*) 0.19. IC_{50} values BSD (µg/ml): (NF54 *Pf*WT) 0.48; (GFP@*cam*) 0.34; (GFP@*gapdh*) 0.54; (GFP@*hsp70*) 0.48. **B**. The growth rate of asexual blood stages in cultures maintained in the semi-automated culture system for a period of 5 days. Cultures were initiated with a parasitemia of 0.5 %.

collected daily from cultures maintained in the semi-automated culture system during a period of 5 days. Growth rates of the transgenic lines were highly comparable to that of NF54 wild type parasites and parasitemias increased from 0.5 to 4-8% during the culture period (**Figure 3B**) also the number of merozoites per schizont in the reporter lines was also comparable to that of the parental *P. falciparum* (NF54) WT line.

GFP expression of GFP@cam, GFP@gapdh and GFP@hsp70 during blood stage development in vitro

We examined GFP expression in the different reporter lines during blood stage development by fluorescence microscopy. Expression of GFP was detectable in merozoites and ring forms of the GFP@*hsp70* and GFP@*gapdh* lines whereas all lines exhibited GFP expression in schizonts and gametocytes (**Figure 4A and S3-5**). This is in agreement with data on transcription of the three genes from which the promoters were used (**Table S2**). Next we more precisely compared the GFP-fluorescence intensity of the different lines by examining the following synchronized stages by flow cytometry: rings (16 hours post invasion; hpi), trophozoites (30 hpi) and schizonts (42 hpi) (**Figure 4B**). Rings (G1), trophozoites (G2) and schizonts (G3) were distinguished based on their DNA content after staining with the DNA-specific dye Hoechst33258. While fluorescence increased during growth of trophozoites of all three lines, the fluorescence intensity did not further increase during schizogony. The GFP@*cam* parasites exhibited the lowest GFP expression with a mean fluorescence intensity (MFI) in trophozoites (MFI 149) only slightly higher that of uninfected cells whereas trophozoites of GFP@*hsp70* parasites and GFP@*gapdh* exhibiting much stronger GFP expression (MFI of 1339 and 1671, respectively). GFP@*hsp70* schizonts showed highest levels of GFP expression (MFI of 3174; **Figure 4B**).

Discussion

Here we report the generation of *P. falciparum* reporter parasites expressing GFP under control of three different *P. falciparum* gene promoters using optimized CRISPR/Cas9 constructs and selection protocol. The introduction of CRISPR/Cas9 based genome editing to *P. falciparum* research has provided a powerful tool, which can be used to better and faster interrogate parasite gene regulation and function [33]. Before CRISPR/Cas9, modifications of the *P. falciparum* genome, such as gene disruption or mutation

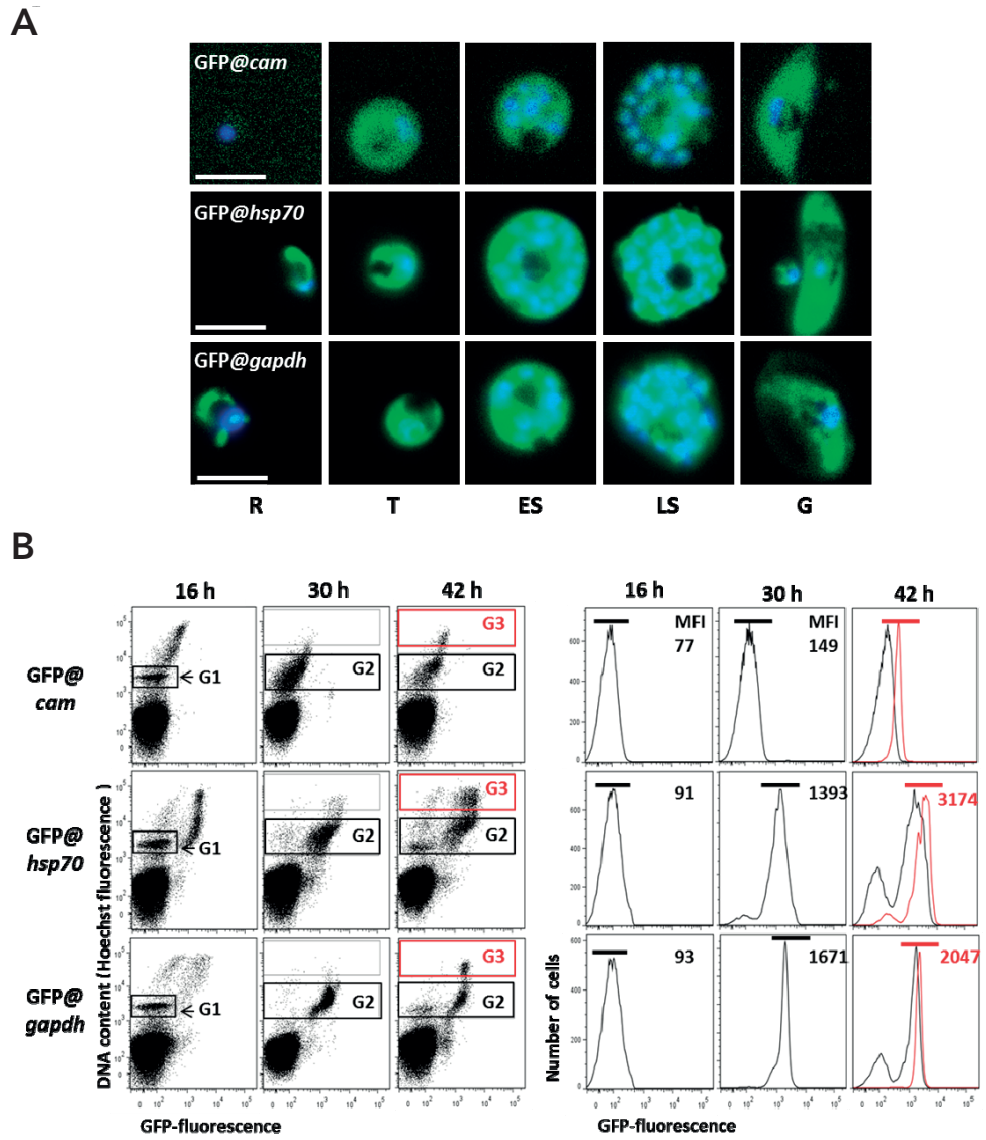


Figure 4. GFP-expression in blood stages of three reporter *P. falciparum* parasite lines (GFP@*cam*, GFP@*gapdh*, GFP@*hsp70*). **A**. Fluorescence microscopy of different blood stages. R: rings; T: trophozoites; ES: early schizonts; LS: late schizonts; G: gametocytes. Nuclei were stained with the DNA-specific dye Hoechst 33342. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (GFP 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain)). In **Figure S3, S4 and S5** the complete set of microscope images are shown. **B**. Fluorescence intensity of rings (16 h), (late) trophozoites (30 h) and schizonts (42 h) as determined by flow cytometry. Infected red blood cells (RBC) were stained with the DNA-specific dye Hoechst 33258 to distinguish infected RBC from uninfected RBC and rings (Gate G1), trophozoites (Gate G2) from schizonts (Gate G3). Left side panels show dot plots of both Hoechst fluorescence intensity (DNA content) and GFP fluorescence intensity. Right side panels show GFP fluorescence intensity from parasites with either G1 and G2 (black) gate or G3 (red). MFI: mean fluorescence intensity and the black- (rings and trophozoites) and red- (schizonts) bar show the region selected to calculate the MFI.

or the introduction of transgenes into the genome, required 1-3 months of continuous culture to select for parasites in which episomally maintained plasmids became integrated into the parasite's genome, either by single or by double cross-over recombination [33]. Further, the process of generating cloned genetically modified and drug-selectable marker-free parasites would typically take 5 months or more to complete [20]. With the methods described here we are able to generate cloned marker-free parasite lines that stably express reporter proteins within a period of 10-12 weeks.

The constructs we have generated can be modified and used in future studies as template constructs to remove or introduce transgenes into the *P. falciparum* genome, for example they could be used to generate reporter lines that express additional (fluorescent/bioluminescent) reporter proteins or used to analyse the regulatory elements that control *Plasmodium* gene expression.

In addition the sgRNA/donor DNA construct can be adapted to permit the introduction of transgenes into other *P. falciparum* genetic loci by adapting the homology regions in the donor plasmid, or the construct can be modified to introduce other transgene (e.g. reporter) expression cassettes under the control of a variety of regulatory promoter and transcription terminator (5'- and 3'- UTR) regions. All the plasmids and *P. falciparum* mutant lines described in this study are available on request.

In the sgRNA/donor plasmid we have placed the selectable marker cassette outside the donor DNA cassette which then does not result in the introduction of the selectable marker into the parasite genome upon repair of the target locus. Hence the locus can be modified without the inclusion of a selectable marker cassette. The same type of constructs can also be used to perform other genetic manipulations, notably gene-disruption and gene-mutations, which can be used to interrogate gene function and importance. As a consequence of the absence of a drug-selectable marker in the genome of the mutants, these constructs can be adapted and used to create rapid successive genetic modifications in the same parasite line. For example, 'doubly' modified parasites that contain both a reporter gene as well as a disrupted (or mutated) gene. Such rodent malaria mutants have been used extensively to analyse the phenotypic consequences of a gene disruption/mutation using a variety of imaging technologies. Moreover, our transfection protocol permits for a more rapid generation of multiply modified parasites since it is possible to further transfect the uncloned population of a modified parasite (after parasite emerge from negative selection). With the high transfection efficiencies we observe in our transfection protocol, combined with the rounds of positive and negative selection, it is possible to create cloned and SM free, doubly transgenic parasites in the same time (~5 months) it would have taken to create a single SM-free genetic modification in *P. falciparum* using conventional approaches. Indeed a cloned *P. falciparum* double gene deletion mutant (PfΔ*mrp1*Δ*mrp2*), which still contained a drug-selectable marker, has been recently reported and it required 3 independent transfections, 2 rounds of cloning, 1 round of SM recycling and took nearly a year to generate [34].

To select for parasites with the donor DNA integrated into the genome we made use of a selectable marker cassette containing a fusion gene of the human dihydrofolate reductase (h*dhfr*) positive selectable marker and a negative selectable marker, the bifunctional protein that combines yeast cytosine deaminase and uridyl phosphoribosyl transferase (y*fcu*). Negative selection based on yFCU expression has been used for both for genetic modification of *P. falciparum* [35] and for rodent malaria parasites [36]. The drug 5-FC efficiently kills parasites that express *y*FCU both in *in vitro* cultures and *in vivo* in laboratory animals. We established that the positive/negative selectable marker (h*dhfr::yfcu*) cassette was functional in *P. falciparum* by transiently transfecting parasites with a plasmid (pLf0033) containing the h*dhfr::yfcu* fusion cassette and obtaining parasites using positive drug (WR99210) selection, followed by the application of negative drug (5-FC) selection that killed the parasites demonstrating that they were sensitive to this drug. In the CRISPR/Cas9 method described here, we apply negative selection to kill parasites that still retain the sgRNA/donor plasmid DNA, enriching for the population of parasites where the donor DNA has integrated into the parasite's genome. Negative selection will also not kill 'wild type' parasites that are still present in the population, i.e. parasites that had lost the donor DNA construct without an integration event. From the ratio of GFP-positive and GFP-negative parasites present in the cultures after negative selection, as well as the results of cloning, we found that percentage of stable transgenic parasites was in excess of 50% and therefore the presence of wild type parasites appeared not to be an obstacle for obtaining the desired mutants. It has also been reported that homologous repair mediated by a donor DNA in *P. falciparum* after CRISPR/Cas9 transfection appears to more efficient than parasite mediated non-homologous end joining repair of DNA, which therefore favours the selection of transgenic parasites over either wild type parasites or parasites that have repaired the double strand break through the introduction of a site specific frame shift [26]. Though it was not necessary in this study, fluorescent reporter parasites can be further enriched or indeed cloned after negative selection by flow/FACS sorting of fluorescent cells.

We generated in this study three different reporter parasite lines principally to select for constitutive and strong promoters that can be used to drive reporter gene expression. In previous studies only a limited number of promoters have been used for genetic modification of *P. falciparum*. For driving transgene expression *eef1*α [17, 18] and *hsp86* [37, 38] have been reported, while *cam* and *hsp86* have been used to drive expression of selectable markers [39]. By comparing RNAseq data of blood stages we selected three genes with expression levels higher than *eef1*α, a promoter that has been previously used to generate GFP-expressing reporter *P. falciparum* lines [17, 18]. We found that the *cam* promoter resulted in relatively weak GFP-expression compared to *gapdh* and *hsp70* which is in agreement with the RNAseq data. GAPDH is an enzyme involved in glycolysis, the main pathway for ATP production in *Plasmodium* [40] and therefore this protein is likely to be expressed throughout the complete life cycle. Indeed proteome analyses of oocysts and sporozoites provide evidence for high abundance of GAPDH in these stages

(PlasmoDB; www.plasmodb.org). Therefore, we believe, that this promoter can be a useful tool to drive transgene expression throughout the complete life cycle.

Currently several CRISPR/Cas9 methods using two plasmid based strategies have been described for *P. falciparum* genetic modification. The constructs described in Ghorbal *et al.* [22] like our study, have both the sgRNA and donor sequences on one plasmid. In contrast to our method, the use of these constructs result in generation of mutants, both deletion mutants and transgenics, that carry a drug selectable marker into their genome. This method has the advantage that there is no need for negative selection to remove parasites that retain the plasmid. While in the Ghorbal *et al.* study marker free point mutation mutants have been generated, it is unclear if their constructs could be used for complete gene deletion or a large genetic insertion without inclusion of a drug selectable marker. As both our and the Ghorbal *et al.* method have the *cas9* gene introduced on a separate plasmid, only one plasmid has to be modified for each subsequent modification (i.e. changing the sgRNA and/or donor sequences) and therefore multiple *P. falciparum* genes can be targeted using a pool of constructs in a single transfection experiment. Consequently, multiple mutants can be obtained from a single transfection experiment. In contrast the method described by Lu *et al.* [26], where the sgRNA and donor sequences are on separate plasmids, requires both plasmids to be modified to create an additional gene modification/disruption. However the strategy described by Lu et al, where the donor DNA is on a separate plasmid, allows for the introduction of larger DNA inserts into the parasite's genome.

When we examined clones from the 3 different transgene mutants 57-66% had the expected genotype. The other clones were WT clones or, more commonly, clones where the donor construct appears to integrate into the parasite genome by single cross-over recombination. While 'single cross-over' parasites retain the h*dhfr*::y*fcu* SM a reduction in sensitivity to 5-FC is expected, due to reduction of h*dhfr*::y*fcu* copies in the genome compared to parasites containing multiple episomal plasmids. A reduction in the selection of the clones with the undesired genotype can be accomplished by increasing the 5-FC concentration during selection and/or by increasing the expression of the h*dhfr*::y*fcu* SM, for example by replacing the promoter of the SM (*Pfhsp86*) with that of a more highly expressed gene (e.g. *Pfhsp70*). This is particularly important when a gene deletion may result in a growth defect (unlike *Pf230p*) and WT parasites may 'over grow' the deletion mutant.

In summary, we have created improved constructs and describe an efficient transfection protocol to create modified *P. falciparum* parasites and these reporter parasites are suitable for further genetic modifications since they are SM-free. Improving the ability to perform genetic manipulations, including making it easier to perform successive gene-deletions and gene-mutations, will not only be of value to interrogate parasite gene function but also for the development of multiple attenuated malaria parasites suitable for vaccination [41].

Materials and methods

Parasites and in vitro cultivation of blood stages

P. falciparum parasites from the NF54 strain [42] were obtained from the Radboud University Medical Center (Nijmegen, The Netherlands). These parasites were used to generate the different transgenic parasite lines. Parasites were cultured following the standard conditions in RPMI-1640 culture medium supplemented with L-Glutamine and 25mM HEPES (Gibco Life Technologies) to which was added 50 mg/L hypoxanthine (Sigma). Culture medium was supplemented with 10% human serum and 0.225% $NaHCO_3$. Parasites were cultured at a 5% hematocrit under 4% O_2, 3% CO_2 and 93% N_2 gas-conditions at 75 rpm at 37°C in a semi-automated culture system in 10ml flasks (Infers HT Multitron and Watson Marlow 520U)[22]. Fresh human serum and human red blood cells (RBC) were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria research and microbiology test for safety). RBC of different donors were pooled every two weeks, washed twice in serum free RPMI-1640 and resuspended in complete culture medium to 50% haematocrit. Human serum of different donors were pooled every 4-6 months and stored at -20°C until required.

Generation of new standard CRISPR/Cas9 constructs

The first Cas9-expressing construct (Cas9; pLf0019), was generated by replacing the drug-selectable marker y*dhodh* of the standard construct pUF1-Cas9 [22] by the *bsd* selectable marker obtained from the pMV-FLPe construct [20] using the restriction enzymes *EcoR*I/*Spe*I. The second construct, containing both the sgRNA as well as the donor DNA sequences (sgRNA/Donor construct; pLf0022), was generated in multiple cloning steps. This construct contains both the sgRNA expression cassette and the selectable marker cassette containing the fusion gene of the positive selectable marker *hdhfr* and the negative selectable marker y*fcu* (yeast cytosine deaminase/uridyl phosphoribosyl transferase [28]. Briefly, the sgRNA-expression cassette under control of the *Plasmodium u6* RNA promoter (PF3D7_1341100) containing the BtgZI adaptor sequence was digested from pL6-eGFP[22] using the restriction enzymes *Nco*I/*Aat*II and cloned in the intermediate plasmid pLf0051. The *P. falciparum hsp86* promoter (PF3D7_0708400) was obtained from JCK-3 plasmid (obtained from Prof. R.W. Sauerwein, Nijmegen, the Netherlands) using the restriction enzymes *Pvu*II/*Sex*AI and was cloned into the *P. berghei* transfection construct pL0034 (RMgm-687; www.pberghei.eu) resulting in plasmid pLf0033. The *P. falciparum hsp86* promoter, replaced the existing *P. berghei eef1*α promoter and was placed upstream of the h*dhfr::* y*fcu* fusion gene (positive/negative selectable marker) and the *P. berghei dhfr/ts* (PBANKA_0719300) transcriptional termination (3'UTR) sequence, which were already present in pL0034 [28]. Subsequently the complete cassette was digested from pLf0033 with the *Stu*I/*Kpn*I restriction sites and cloned into pLf0051 with *EcoR*V/*Kpn*I, resulting in the final construct pLf0022. This construct contains additional restriction sites for

introducing homology/targeting sequences to target any gene of interest such as *Stu*I/*Sac*II and *Apa*I/*Hind*III (see below).

P. falciparum 230p (Pf230p) targeting constructs

Constructs were designed to target the *Pf230p* locus (PF3D7_0208900) in the *P. falciparum* genome. To generate the *Pf230p* targeting vectors, plasmid pLf0022 (see above) was modified introducing two homology regions targeting *Pf230p*. Homology region 1 (HR1) was amplified using primers P1/P2 and homology region 2 (HR2) with P3/P4 from *P. falciparum* NF54 genomic DNA (see **Table S1** for primer details). HR1 was cloned in pLf0022 using restriction sites *Stu*I/*Sac*II and HR2 using *Apa*I/*Hind*III, resulting in intermediate plasmids CM162 and CM163. A guide sgRNA (sgRNA2) sequence for *Pf230p* was identified using the Protospacer software (alpha version; https://sourceforge.net/projects/protospacerwb/files/Release/) and was amplified using the primers P7/P8. This sgRNA was selected based on the best off targets hits score throughout the genome given by Protospacer and the total number of mismatches of the sgRNA with respect to the PAM site. A 20 bp guide sgRNA, surrounded by 15 bp vector specific DNA necessary for InFusion cloning (HD Cloning Kit; Clontech), was annealed and used to replace the BtgZI adaptor as previously described [22], resulting in construct pLf0024. The construct was digested with *Bln*I and *Nru*I to evaluate the successful cloning of the sgRNA and later confirmed by Sanger sequencing using primers P9/P10.

The generation of the three *Pf230p* targeting constructs that contain the *gfp* gene under different promoters were constructed in multiple cloning steps. The promoters were selected based on published transcript levels of their genes in asexual blood stages (RNA seq data available in PlasmoDB, www.plasmodb.org). The promoters of the following genes were selected *cam* (*calmodulin*; PF3D7_1434200); *gapdh* (*glyceraldehyde-3-phosphate dehydrogenase*; PF3D7_1462800) and *hsp70* (*heat shock protein 70*; PF3D7_0818900). The *cam* promoter was amplified from NF54 genomic DNA using primers P11/P12 and cloned in the intermediate plasmid pLf0052 using the enzymes *Aat*II/*Bam*HI. This plasmid contains the *gfp* expression cassette with the *P. falciparum cam* promoter region and the 3' UTR region from the *calmodulin* gene from *P. berghei* ANKA (PBANKA_1010600), which was previously amplified from intermediate plasmid Plf0012 using primers P17/P18. The *gfp@cam* expression cassette was obtained by digestion with *Apa*I/*Pvu*II and cloned into plasmid pLf0024 (see above) using restriction sites *Apa*I/*EcoR*V, resulting in the final *gfp@cam* construct pLf0026.

The *gapdh* promoter was amplified from NF54 genomic DNA using primers P13/P14 and used to replace the *cam* promoter by the *gapdh* promoter in intermediate plasmid pLf0052 using the restriction sites *Aat*II/*BamH*I. The complete *gfp@gapdh* expression cassette from this plasmid was digested with *Apa*I/*Pvu*II and cloned into pLf0024 (see above) using restriction sites *Apa*I/*EcoR*V, resulting in the final *gfp@gapdh* construct pLf0032.

The complete *gfp@hsp70* expression cassette was obtained by digestion from the intermediate plasmid pLf0053 using restriction enzymes *Apa*I/*Pvu*II and cloned into pLf0024 (see above) using restriction sites *Apa*I/*EcoR*V resulting in the final *gfp@hsp70* construct pLf0035. The *hsp70* promoter was amplified from NF54 genomic DNA using primers P15/P16. For the 3'UTR of the *gfp@hsp70* expression cassette the 3'UTR of the gene encoding the *histidine-rich protein II* (PF3D7_0831800) was amplified with primers P19/P20 from the plasmid pHHT-FRT-(GFP)-Pf52 [20].

The *gfp@cam* and *gfp@gapdh* plasmids were created using the intermediate plasmid pLf0052, resulting in the same orientation of the GFP expression cassette and the same 3' UTR (*calmodulin* gene) whereas the *gfp@hsp70* plasmid was created using an intermediate plasmid pLf0053 which resulted in the reporter cassette in a reverse orientation and the *histidine-rich protein II* 3'UTR.

All PCR amplifications were performed with high-fidelity Phusion DNA polymerase (New England Biolab) following the recommended protocols, except for the promoters (*cam, gapdh* and *hsp70*) that were amplified with KOD Hot Start polymerase (Novagen) under standard conditions. All cloning and plasmid amplifications were done in *Escherichia coli*, XL10-Gold Ultracompetent Cells (Stratagene). Details of the primer sequences are shown in **Table S1**.

Transfection and selection of transgenic parasites

Plasmids for transfection were isolated from 250 ml cultures of *Escherichia coli*, XL10-Gold Ultracompetent Cells (Stratagene) by maxi-pep (using HiSpeed® Plasmid Maxi Kit (Qiagen®)) to generate the 50 µg of DNA used per transfection. Transfections were performed using ring stage parasites obtained from cultures with a parasitemia of 6 – 15% that were synchronized by 5% D-sorbitol treatment 2 days before transfection [43]. Infected RBC were pelleted by centrifugation (1150*g*, 5 min.) and 300 µl of the pelleted cells were transferred to a 0.2 cm cuvette and mixed with ~50 µg of each circular plasmid (Cas9 construct and sgRNA/Donor construct) in 100 µl cytomix [44]. Electroporation was performed with a single pulse (310 V and 950µF) in the Biorad Gene Pulser Xcell electroporator (including CE- and PC module). After transfection cells were immediately transferred in a 10 ml culture flask and cultures were maintained under standard conditions in the semi-automated culture system (see above).

Selection of transfected parasites was performed by applying 'double' positive selection 24 h after transfection using the drugs WR99210 (2.6 nM) and BSD (5 µg/ml). For WR99210 100 µl of a stock solution (2.6 µM) was added to 100 ml complete culture medium resulting in a final concentration of 2.6 nM. To prepare the WR99210 stock-solution WR99210 was dissolved in DMSO (100mM). For BSD 50 µl of a stock solution (10mg/ml) was added to 100 ml complete culture medium resulting in a 5 µg/ml final concentration. The drug pressure was maintained until thin blood-smears were parasite-positive (usually after 14 to 26 days). Positive selection will select for the parasites that were transfected successfully

with both plasmids (Cas9 and sgRNA/Donor constructs). Subsequently, both drugs were removed from the cultures for 2-4 days, followed by applying negative selection by addition of 5-Fluorocytosine (5-FC; 130 µl of a stock solution (0.77 mM) in 100 ml complete medium with a final concentration of 1 µM; [45]) in order to eliminate parasites that retained the sgRNA/Donor construct as episomal plasmid and enriching for those transfected parasites where the donor DNA had integrated into the genome. Negative drug pressure was maintained until thin blood-smears were parasite-positive (usually after 7 days). During both positive and negative selection period, parasites were analysed for GFP expression by fluorescence microscopy (see below) to determine the ratio of wild type and mutant parasites present in the population. After negative selection parasites were harvested from cultures with 4 to 10% of parasitemia for genotyping by diagnostic PCR and Southern analysis (see below).

Cloning of transgenic parasites

Based on the percentage of GFP-positive parasites in cultures after negative selection and PCR confirmation of double cross-over integration the transgenic parasites were cloned by the method of limiting dilution as previously described [46] with minor modifications. Briefly, infected RBC from cultures with a 4% to 10% parasitemia were diluted with uninfected RBC to 10^5 infected RBC/100 µl in 2 ml culture medium (1% hematocrit and 20% serum). Serial dilutions were then performed with uninfected RBC in complete medium (1% hematocrit and 20% serum) and cultured in a total volume of 100 µl incubated in 96 well plates, resulting in 8 rows with the following numbers of parasites per well in the different rows: 100, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15. Plates were incubated in a Candle Jar at 37°C and culture medium was changed every other day. Every 5 days RBC were added resulting in an increase of the hematocrit from 1% to 5%. Between days 10-14 samples were collected for thick smear analysis from the rows with the highest numbers of infected RBC/well; 50 µl medium was removed and from the remaining culture 5 µl was used directly for preparing thick smears. At day 21 thick smears were made from all rows. Clones were selected from dilutions/row with less than 30% of the wells parasite positive. These clones were transferred in 10 ml culture flasks at 5% hematocrit under standard culture conditions (see above) in the semi-automated culture system for collection of parasites for further genotype and phenotype analyses (see below).

Genotype analysis of cloned transgenic parasite lines

For genotyping by diagnostic PCR and Southern analysis were performed from material isolated from infected RBC obtained from 10ml cultures (parasitemia 3 - 10%), pelleted by centrifugation (1150 *g*; 5 min.). RBC were then lysed with 5-10 ml of cold (4°C) erythrocyte lysis buffer (10x stock solution 1.5 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M Na_2EDTA; pH 7.4; [43]) and parasites were treated with RNAse and proteinase-K before DNA isolation by standard phenol-chloroform methods. Correct integration of the donor construct was analysed by standard and long-range PCR (LR-PCR). In brief, for the GFP*@cam* and GFP*@hsp70* expression cassette integration was confirmed by LR-PCR using the primers P23/P28 and for GFP*@gapdh* the integration was confirmed using the P30/P26 primers (see **Table S1** for details of the primers). The LR-PCR fragments were amplified using KOD Hot start polymerase following standard conditions with an annealing temperature of 53.5°C for 15 s and an elongation step of 68°C for 9 min. All other PCR settings were according to manufacturer's instructions.

Southern blot analysis was performed with genomic DNA digested with *Xho*I and/or *Spe*I restriction enzymes (4 h at 37°C) in order to confirm integration of the expression cassette into the *Pf230p* locus. Digested DNA was hybridized with probes targeting the *Pf230p* homology regions, amplified from NF54 genomic DNA by PCR using the primers P1/P2 for HR1 and P3/P4 for HR2 respectively.

Phenotype analysis of parasites

The growth rate of asexual blood stages of clones of the three transgenic lines was monitored in 10 ml cultures maintained in the semi-automated culture system under standard culture conditions (see above). Briefly, a 0.5% parasitemia culture was established in complete culture medium at a haematocrit of 5%. Medium was changed twice daily and the culture maintained for a period of 5 days without refreshing RBC. For determination of the course of parasitemia, triplicate samples of 100 µl were collected daily from all cultures and cells pelleted by centrifugation (9485 *g* ; 30s). The culture medium was then removed and cells were washed twice in 1X PBS before and after fixation with 0.25% glutaraldehyde (30 min. at 4°C). Fixed RBCs were stained with the DNA-specific dye Hoechst33258 in 1 ml of PBS by adding 4 µl of a 500 µM stock-solution (final concentration 2 µM). Samples were stained for 1hr at 37°C in the dark and analysed by FACS [47].Hoechst-fluorescence intensity of stained cells was measured using an LSRII flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data was analysed using FlowJo software (Treestar, Ashland, OR, USA). At least 50 000 cells were analysed per sample and the parasitemia was determined by FACS using an UV laser (355 nm) and band pass filter 450/50 nm [47] and examining the number of Hoechst-positive and Hoechst-negative cells. RBCs were selected by gating on Forward and Side Scatter parameters (FSC and SSC, respectively). Doublets are excluded by using FSC-Area and FSC-height parameters.

Drug-sensitivity of asexual blood stage parasites from cloned lines of the three transgenic lines was analysed as described previously [20], with the following modifications. Infected RBCs (0.1%-0.5% parasitemia) at 1% of haematocrit were cultured in 96-wells culture plates in a Candle Jar (in complete medium and 20% human serum). To each well containing 100 µl of the infected RBC culture was added another 100 µl of culture medium containing different concentrations of BSD or WR99210 with concentrations ranging from 0.1 to 1µg/ml BSD or from 0.01 to 100 nM WR99210; each drug concentration was performed in triplicate wells). Serial dilutions were made from stock-solutions of 1 mg/ml and 1 mM of BSD and WR99210, respectively. Medium of the cultures was changed daily.

Determination of the parasitemia in the culture wells was determined at 72 h after start of the cultures by flow cytometry. Briefly, cells were pelleted by centrifugation (9485 *g*, 30 s) and cells were washed twice in 1XPBS before and after fixation with 0.25% glutaraldehyde (30 min. at 4°C). Fixed RBC cells were stained with the DNA-specific dye Hoechst 33258 in 1 ml of PBS by adding 4 µl of a 500 µM stock-solution (final concentration 2 µM). Samples were stained for 1 h at 37°C in the dark and analysed by flow cytometry [47]. Determination of parasitemia (= percentage of infected RBC) by flow cytometry was determined as described above and was analysed using GraphPad Prism software (GraphPad software, Inc., US). Parasite survival is defined as the percentage of infected RBC in drug-treated wells divided by the percentage of infected RBC in non-treated wells multiplied by 100. For calculation of the survival curves, the mean fluorescence intensity value of samples with the highest drug concentration (i.e. with maximum inhibition of growth) is subtracted from the mean fluorescence intensity value of the samples with the other drug concentrations and the control samples without drug. The mean parasitemia of the control samples without drug is set at 100% and the mean parasitemia of the highest drug concentration is set at 0% for calculation of the parasite survival. Growth inhibitory curves and statistical analysis of the data is performed using the GraphPad Prism software. The non-linear regression function for sigmoidal dose-response (variable slope) of the GraphPad Prism software is used to calculate the (best-fit) EC_{50} values.

GFP expression in different blood stages was analysed by standard fluorescence microscopy. In brief samples of approximately 200 µl were collected from 10 ml infected cultures with parasitemias between 4 and 10%. The RBC samples were stained with the DNA-specific dye Hoechst 33342 by adding 4 µl of a 500 µM stock-solution to a final concentration of 10 µM for 20 min. at 37°C. Five µl of the preparation was mounted on a microscopic slide under a cover slip to visualize the parasites by Hoechst and GFP fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software and with the following exposure times: GFP 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain).

The relative GFP-fluorescence intensity of different asexual blood stages was analysed by flow cytometry. Triplicate samples of 100 µl of infected RBC were collected from cultures that had been synchronized with sorbitol and cultured in the semi-automated *in vitro* system. Samples were collected at 30 and 42 h after synchronization and resuspended in 1 ml of culture medium containing 5% serum. Cells were stained with the DNA-specific dye Hoechst33258 by adding 20 µl of a 500 µM stock-solution to a final concentration of 10 µM [47]. Staining was performed for 30 min. at 37°C. GFP and Hoechst fluorescence intensity was determined using a LSRII flowcytometer (Becton Dickinson, Mountain View, CA, USA) and the data was analysed using FlowJo software (Treestar, Ashland, OR, USA). 100.000 cells were analysed per sample and RBC were selected by gating on FSC and SSC. Doublets are excluded by using FSC-Area and FSC-height parameters. Excitation of cells for Hoechst33258 was performed with a UV laser (355 nm) and band pass filter 450/50 nm and for GFP with a blue laser (488 nm) and a band pass filter of 530/30 nm. The GFP fluorescence intensity was determined of the haploid blood stages (rings and trophozoites; Gate 1) and polyploid blood stages (schizonts; Gate 2). Haploid and polyploid blood stages were distinguished based on Hoechst-fluorescence intensity [47]. Data generation was performed using the FACS DIVA software (Becton Dickinson) and analysed with FlowJo software.

Acknowledgements

The pUF1-Cas9 and pL6-eGFP plasmids were obtained from Jose-Juan Lopez-Rubio (Paris, France) and the JCK-3, pHHT-FRT-(GFP)-Pf52 plasmids and *P. falciparum* (NF54) from Prof. R.W. Sauerwein (Nijmegen The Netherlands). Catherin Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). Takashi Imai was, in part, supported by Uehara Memorial Foundation grant.

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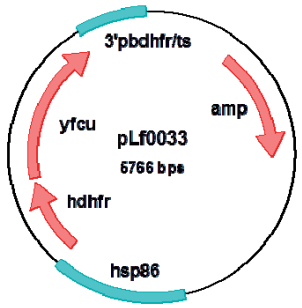
Supplementary Data

A



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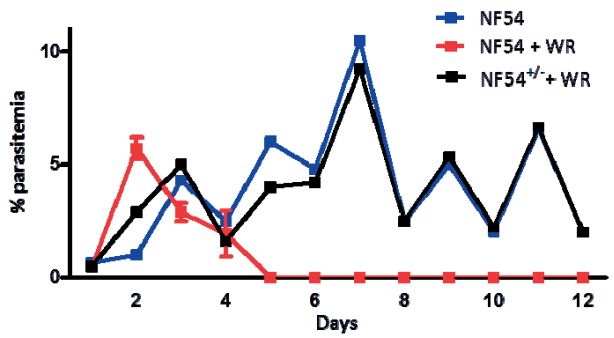

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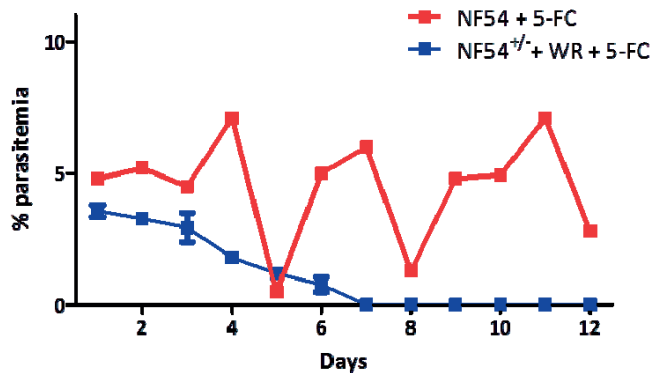
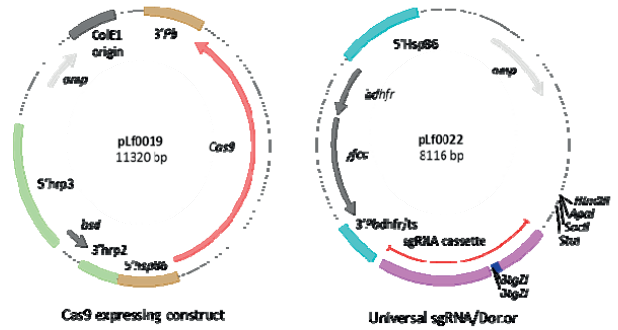


Supplementary figure 1. Drug sensitivity of *P. falciparum* parasites expressing the h*dhfr*::y*fcu* fusion cassette. **A**. Vector map of pLf0033, expressing the h*dhfr*::y*fcu* SM cassette, used for transient transfection. **B.** Growth of NF54 blood stage parasites in the absence or presence of the positive drug, WR99210 (WR; 2.6nM final concentration). WT *P. falciparum* NF54 parasites (NF54) were episomally transfected with the plasmid pLf0033, encoding a positive/negative drug selection h*dhfr*::y*fcu* fusion cassette (NF54 $^{+/-}$plasmid) and selected under positive (WR) selection. Cultures were diluted to ~0.5% parasitemia with fresh erythrocytes when parasitemia reached 5-10%. **C**. Episomally transfected *P. falciparum* parasites (NF54 $^{+/-}$plasmid), which were initially selected under positive (WR) selection, and WT *P. falciparum* NF54 parasites were subjected to negative (5-FC 1µM final concentration). Cultures were diluted to ~0.5% with fresh erythrocytes when parasitemia reached 5-10%.

A



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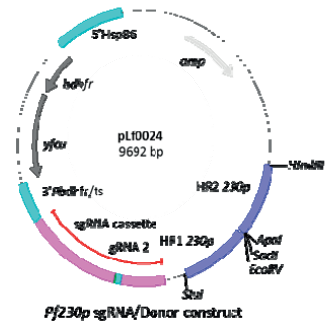
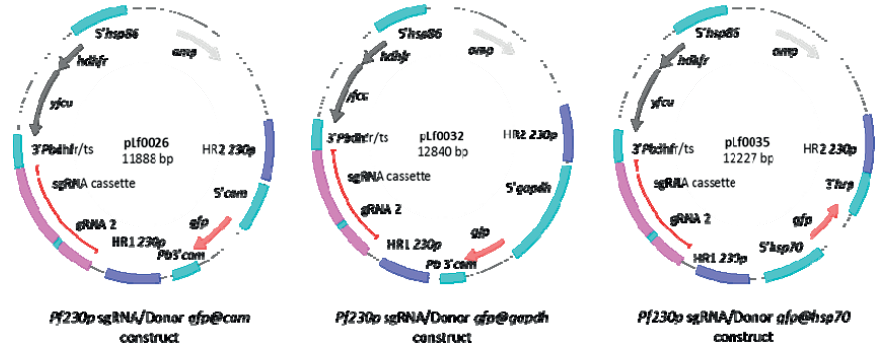
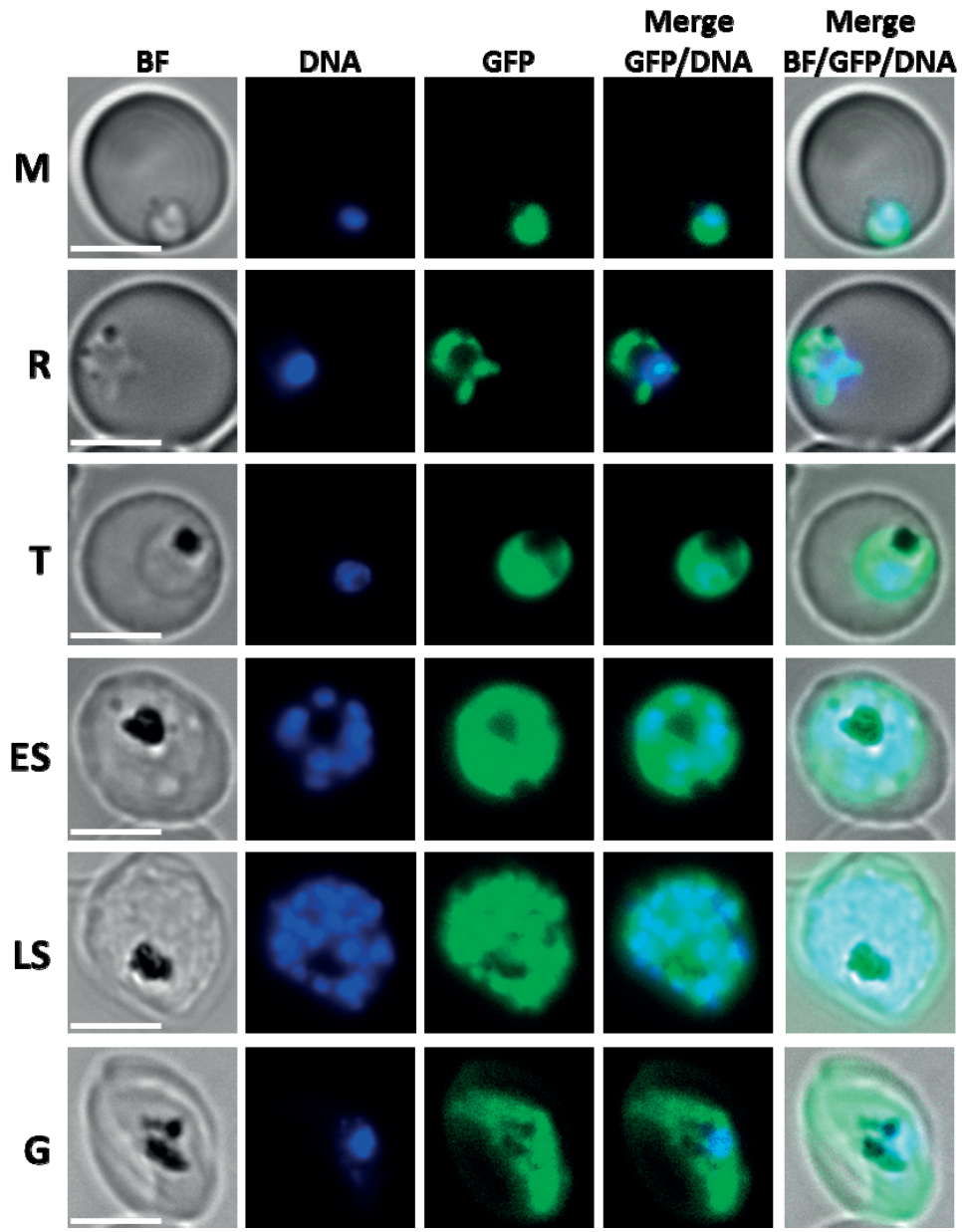
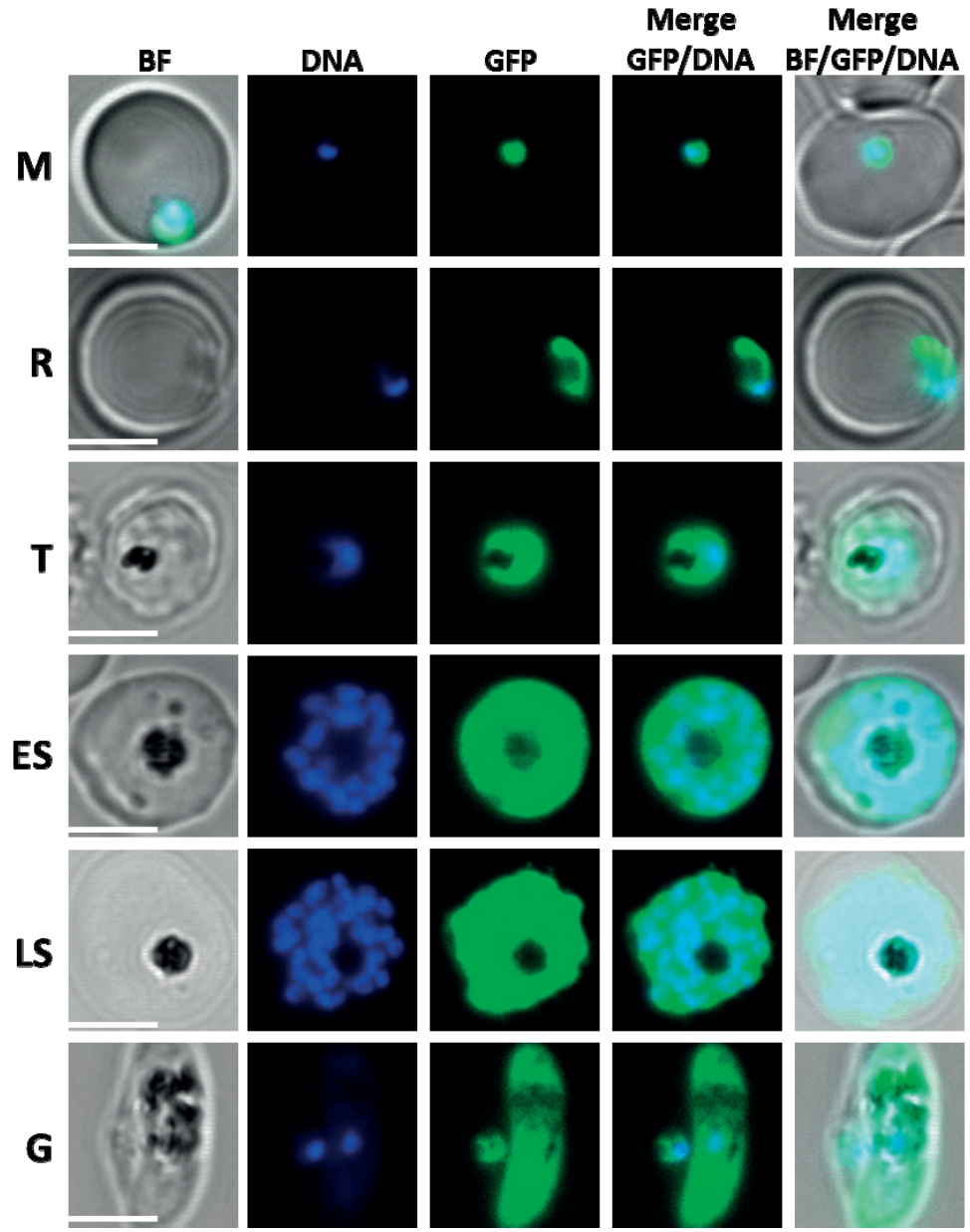




Supplementary figure 2. A. Basic constructs: pLf0019 for Cas9-expression construct with the *bsd* selectable marker; pLf0022 sgRNA/donor construct and pLf0024 for targeting the *Pf230p* locus. **B.** Constructs used for introduction of the GFP-expression cassettes into the *P. falciparum* genome: pLf0026 for *gfp@cam*, pLf0032 for *gfp@gapdh* and pLf0035 for *gfp@hsp70* into *Pf230p*.

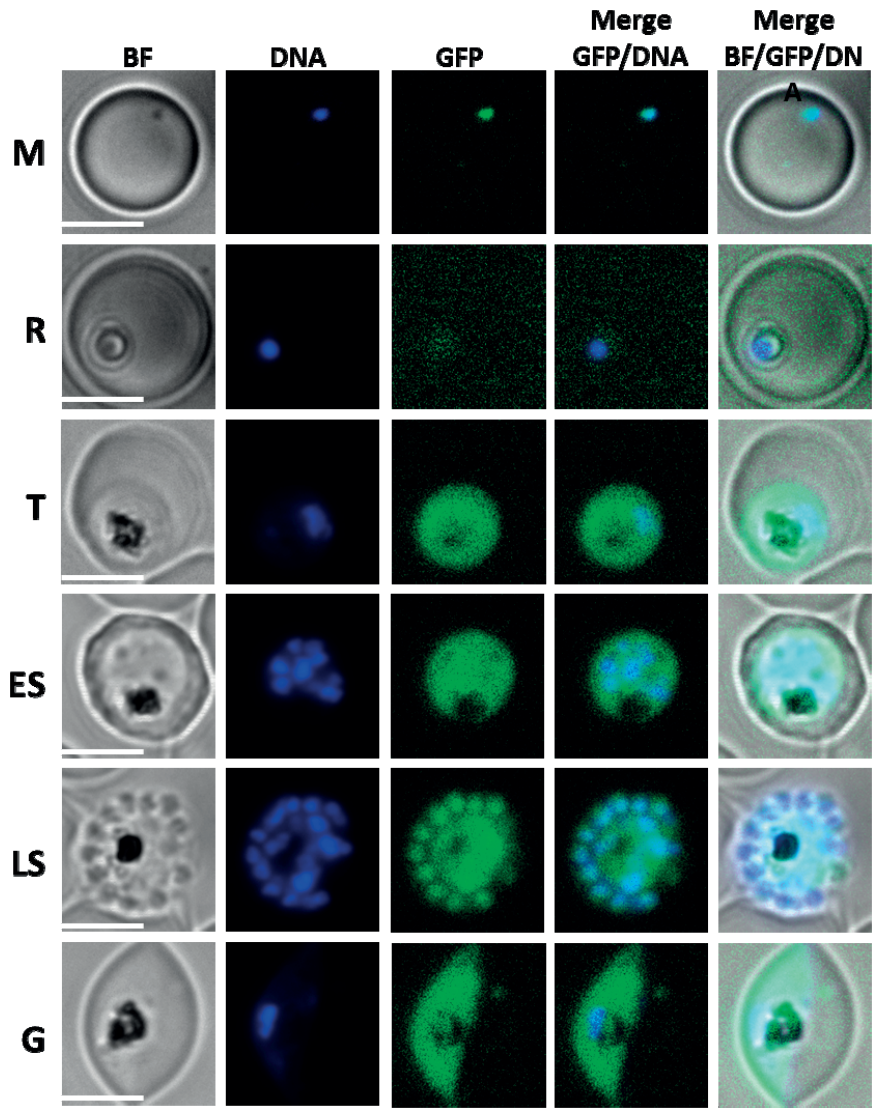


Supplementary figure 3. Fluorescence microscopy of GFP@*gapdh* blood stages parasites. R: rings; T: trophozoites; ES: early schizonts; LS: late schizonts; G: gametocytes. Nuclei were stained with the DNA-specific dye Hoechst 33342. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (GFP 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain)).



Supplementary figure 4. Fluorescence microscopy of GFP@*hsp70* blood stages parasites. R: rings; T: trophozoites; ES: early schizonts; LS: late schizonts; G: gametocytes. Nuclei were stained with the DNA-specific dye Hoechst 33342. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (GFP 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain)).

3



Supplementary figure 5. Fluorescence microscopy of GFP@*cam* blood stages parasites. R: rings; T: trophozoites; ES: early schizonts; LS: late schizonts; G: gametocytes. Nuclei were stained with the DNA-specific dye Hoechst33342. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (GFP 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain)).

Supplementary table 1. List of primers used in this study.

Primer ID	Leiden code	Gene ID	Sequence	Enzymes	Product (bp)	Description
***Pf230p* deletion Homology Regions**						
P1	7865	PF3D7_0208900	TAATTAGGCCTGCCGGCCATATTTATGTGACTTCTTAAAC	*StuI*/*NaeI*	848	Forward HR 1 *pf230p*
P2	7867	PF3D7_0208900	TTCCTCCGCGGGATATCCAACCTTCTATTGGATTC	*SacII*/*EcoRV*		Reverse HR 1 *pf230p*
P3	7869	PF3D7_0208900	TTATTGGGCCCGTCGACGTTGATAAGGATAGTGTTTCAG	*ApaI*/*SalI*	867	Forward HR 2 *pf230p*
P4	7871	PF3D7_0208900	TCCTTAAGCTTTACGTAGGATTAATATTCCCATTAGG	*HindIII*/*SnaBI*		Reverse HR 2 *pf230p*
sgRNA						
P7	7882	PF3D7_0208900	TAAGTATATAATATTGAATATTATTCTAATGATAAGTTTTAGAGCTAGAA		50	Forward sgRNA 2
P8	7883	PF3D7_0208900	TTCTAGCTCTAAAACTTATCATTAGAATAATATTCAATATTATATACTTA			Reverse sgRNA 2
Promoters						
P11	7896	PF3D7_1434200	GTAATAGACGTCGGCCAAATAAGAAATATAAT	*AatII*	704	Forward *calmodulin* promoter
P12	7897	PF3D7_1434200	ATCTGGATCCGATATATTTCTATTAGGTATTTATTATT	*BamHI*		Reverse *calmodulin* promoter
P13	7894	PF3D7_1462800	AACTATGACGTCGCTATGAAAAACATGGGTGTG	*AatII*	1657	Forward *gapdh* promoter
P14	7895	PF3D7_1462800	AAATAGGATCCGAAAAGAATTAAAAAGCCGAAG	*BamHI*		Reverse *gapdh* promoter
P15	7733	PF3D7_0818900	AATAAGACGTCCGCATAAATATCTGGTGAAATACAAAC	*AatII*	968	Forward *hsp70* promoter
P16	7734	PF3D7_0818900	AAATTCTCGAGGAACCTTTTGCACTAGCCAATTTTTC	*XhoI*		Reverse *hsp70* promoter
3′ UTRs						
P17	7589	PBANKA_1010600	TTATTCAATTGACCGGTGGCCGCGACTCTAGAATTAT	*MunI*/*AgeI*	448	Forward 3′ *calmodulin* UTR *P. berghei*
P18	7706	PBANKA_1010600	AATTACCCGGGTCGCGACGGTACCGACCATATAAGAATTAAC	*SmaI*/*NruI*		Reverse 3′ *calmodulin* UTR *P. berghei*
P19	7735	PF3D7_0831800	TTGTTTCTAGAGTTAACCTAGGGAAGTATATGAG	*XbaI*	638	Forward 3′ *histidin rich protein* UTR
P20	7736	PF3D7_0831800	TAAATGGGCCCCTTCGAATTCTGGATTTAATAAATATG	*ApaI*		Reverse 3′ *histidin rich protein* UTR
Genotyping						
P21	7471	PF3D7_0405300	AGCCGCGGCATGGAGAAGGGTTCTATTTTATCG	*SacII*	5383	Forward primer DNA control PCR *lisp2*
P22	7470	PF3D7_0405300	AACGCTAGCTTCCGGATCGCTGTCTTTAC	*NheI*		Reverse primer DNA control PCR *lisp2*
P23	7965		GAACCCAAAGATTGTTTTTCAC			Forward Integration 1 GFP@*cam* Forward integration 1 GFP@*gapdh* Forward LRPCR GFP@*cam* Forward LRPCR GFP@*hsp70*
P24	2547		CGAGCTGGACGGCGACGTAAAC			Reverse Integration 1 GFP@*cam* Reverse integration 1 GFP@*gapdh* Forward *gfp*
P25	5515		GCATGGACGAGCTGTACAAG			Forward Integration 1 GFP@*hsp70*
P26	7966		GTAGATGAACTATTTAATAATACATGTGATTTAG			Reverse Integration 1 GFP@*hsp70* Reverse LRPCR GFP@*gapdh*
P27	2548		CAGCAGGACCATGTGATCGCG			Reverse *gfp*
P28	7967		GTATCTTTTAAATAATACGGTGTAACATC			Reverse LR-PCR GFP@*hsp70* Reverse LR-PCR GFP@*cam*
P30	7964		CTTATGAACGTACATCAGGAGAAG			Forward LR-PCR GFP@*gapdh*

Supplementary table 2. Transcript abundance (RNAseq RPKM values) during asexual blood stage development of four genes. Data obtained from PlasmoDB (www.plasmodb.org; published in Otto *et al.* (2010) *Mol. Microbiol.* 76(1):12-24).

	eef1-a PF3D7_1357000		cam PF3D7_1434200		gapdh PF3D7_1462800		hsp70 PF3D7_0818900	
Time point	**Non Unique***	**Unique**	**Non Unique***	**Unique**	**Non Unique***	**Unique**	**Non Unique***	**Unique**
0 h	1.766	215	0	335	0.000	1.413	1	3.668
8 h	2.143	283	0	226	0.000	2.519	1	3.211
16 h	2.548	297	0	178	0.092	2.663	3	4.100
24 h	5.909	642	0	256	0.000	6.900	4	6.378
32 h	4.329	581	0	561	0.000	12.811	1	4.975
40 h	1.435	132	0	366	0.177	3.201	1	1.188
48 h	1.371	130	0	330	0.158	3.086	1	1.165

* Transcript levels of reads per kilobase of exon model per million mapped reads (RPKM). Non-Unique sequences are shown to indicate the maximum expression potential of the analysed gene. *eef1-a* (*elongation factor 1*α); *cam* (*calmodulin*); *gapdh* (*glyceraldehyde-3-phosphate dehydrogenase*); *hsp70* (*heat shock protein 70*).

CHAPTER 4

The *Plasmodium falciparum* male gametocyte protein P230p, a paralog of P230, is vital for ookinete formation and mosquito transmission

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Scientific Reports. 2018 Oct 8;8(1):14902.
doi: 10.1038/s41598-018-33236-x

Abstract

Two members of 6-cysteine (6-cys) protein family, P48/45 and P230, are important for gamete fertility in rodent and human malaria parasites and are leading transmission blocking vaccine antigens. Rodent and human parasites encode a paralog of P230, called P230p. While P230 is expressed in male and female parasites, P230p is expressed only in male gametocytes and gametes. In rodent malaria parasites this protein is dispensable throughout the complete life-cycle; however, its function in *P. falciparum* is unknown. Using CRISPR/Cas9 methodology we disrupted the gene encoding *Pfp230p* resulting in *P. falciparum* mutants (*Pf*Δ*p230p*) lacking P230p expression. The *Pf*Δ*p230p* mutants produced normal numbers of male and female gametocytes, which retained expression of P48/45 and P230. Upon activation male *Pf*Δ*p230p* gametocytes undergo exflagellation and form male gametes. However, male gametes are unable to attach to red blood cells resulting in the absence of characteristic exflagellation centres *in vitro*. In the absence of P230p, zygote formation as well as oocyst and sporozoite development were strongly reduced (>98%) in mosquitoes. These observations demonstrate that P230p, like P230 and P48/45, has a vital role in *P. falciparum* male fertility and zygote formation and warrants further investigation as a potential transmission blocking vaccine candidate.

Introduction

The s48/45 domain 6-cysteine (6-cys) family of *Plasmodium falciparum* proteins is a small family with 14 members that show stage-specific expression throughout the parasite life cycle and most members localize at the parasite surface [1]. Most members have critical roles in parasite development, either in the vertebrate host or in the mosquito vector, and several members are leading targets for malaria vaccines. Four members, P48/45, P47, P230 and P230p are specifically expressed in the sexual stages of the parasite and are encoded by 2 paralogous pairs of genes. Immune responses directed against the proteins P48/45 and P230 can prevent parasite transmission through the mosquito and these antigens are being actively pursued as so called transmission blocking vaccines [2-4]. Using specific antibodies and rodent and human parasite mutants lacking P48/45 and P230 it has been shown that both proteins are crucial for efficient transmission through mosquitoes [5-7]. In the rodent parasite *P. berghei* these proteins are expressed at the surface of male gametes and are critical for attachment of male gametes to female gametes [5]. In *P. falciparum* these proteins are expressed in both male and female gametocytes/gamete [8-10]. *P. falciparum* P230 has been shown to play a critical role in interactions of male gametes with red blood cells (RBC). In mutants lacking P230 expression the characteristic clusters of uninfected red blood cells that form around male gametes, so-called exflagellation centres, are absent [7]. This 'loss of exflagellation centres' phenotype was not observed for equivalent *P. berghei* mutants lacking P230 expression [5]. In *P. berghei* and *P. falciparum* the paralog of P48/45, the female specific P47 protein is located on the surface of female gametes, zygotes and ookinetes [11]. P47 is important in protecting ookinetes from the mosquito's complement-like immune response in both rodent and human malaria species [12-14]. In addition, *P. berghei* P47 plays an essential role in the attachment and recognition of the female gamete by the male gamete [5, 12]. In contrast, *P. falciparum* P47 does not play such a crucial role in gamete fertilization [11]. These observations indicate that differences exist in the precise function of the sex-specific 6-Cys members between human and rodent malaria species.

In two rodent *Plasmodium* species the paralog of P230, the male-specific P230p protein, appears to be dispensable throughout the parasite's complete life cycle [5, 15, 16]. *P. berghei* and *P. yoelii* mutants lacking expression of P230p can develop in the vertebrate host and in the mosquito vector without a discernible phenotype and *p230p* knock-out parasites manifest a wild type parasite phenotype. Consequently, as P230p is non-essential, the *p230p* gene is the most frequently locus used to introduce additional transgenes into rodent malaria parasite genomes [15].

The function of P230p of human malaria parasites is unknown but, like in rodent parasites, *P. falciparum* P230p is male specific [5, 8-10, 17-19]. Recently we generated transgenic *P. falciparum* parasites where we disrupted the *p230p* gene by introducing transgenes into this locus using adapted CRISPR/Cas9 methodology [20]. These *Pf*Δ*p230p* parasites show normal blood stage growth and are able produce gametocytes. In this

study, we analysed the phenotype of the sexual stages and subsequent developmental mosquito-stages of these *PfΔp230p* parasites. We show that *P. falciparum* P230p has a vital role during mosquito transmission, which is in strong contrast to P230p of rodent malaria parasites. *PfΔp230p* male and female gametes retain P48/45 and P230 expression on male gametocytes. However, like *P. falciparum* mutants lacking P230, the capacity of *PfΔp230p* male gametes to bind to RBC is strongly reduced. In the absence of P230p expression, ookinete and oocyst development in *Anopheles stephensi* mosquitoes is almost absent. These observations identify P230p as one of a limited number of gamete-specific proteins critical for *P. falciparum* transmission.

Results

P. falciparum mutants lacking expression of P230p (PfΔp230p-1 and PfΔp230p-2)

We recently generated two transgenic *P. falciparum* mutants, where different GFP-expression cassettes had been introduced into the *p230p* gene locus using CRISPR/Cas9 technology [20]. In these mutants, GFP@cam and GFP@hsp70, GFP expression is driven by promoters of two different genes, *calmodulin* and *hsp70*. The introduction of GFP-expression cassette resulted in the disruption of the *p230p* gene including removal of 259 bp of the *p230p* coding sequence (**Figure 1A**). We name here these two mutants *PfΔp230p-1* (GFP@cam) and *PfΔp230p-2* (GFP@hsp70). Correct integration of the GFP-expression cassettes in the *p230p* locus has been demonstrated by diagnostic PCR and Southern analysis [20]. The *p230p* gene is a paralogue of the *p230* gene, which is located directly down-stream of *p230p*. To show that the integration of the GFP-expression cassette disrupted only the *p230p* gene and did not alter the *p230* locus, we performed additional Southern analysis of *Sph*I/*Spe*I restricted DNA of WT and *PfΔp230p-1*, using a probe targeting the *p230p* gene-homology region (867 bp) and a probe specific for 745 bp of the *p230* open reading frame. The first probe hybridized to expected DNA fragments which differ in size in WT and *PfΔp230p-1* (4141 bp and 3755 bp, respectively), and the second probe hybridized to a fragment of the same size (5747 bp) in WT and *PfΔp230p-1* (**Figure 1A, B;** see **Table S1** for primer sequences). These hybridisation results indicate the specific targeting of the *p230p* locus by the CRISPR/Cas9 constructs.

P230p is expressed exclusively in male gametocytes as has been demonstrated by immunofluorescence and PCR analyses as well as RNAseq and proteome analyses of separated *P. falciparum* male and female gametocytes [8-10, 17-19]. In contrast, the paralog P230 and P48/45 have comparable expression levels in both males and females parasites ([8-10]; **Table S2**). To demonstrate that gametocytes of *PfΔp230p-1* and *PfΔp230p-2* were deficient in *p230p* expression, we performed RT-PCR and Northern blot analysis using mRNA isolated from gametocyte cultures (**Figure 1C**). No *p230p* transcripts were detected by RT-PCR in gametocytes from either *PfΔp230p-1* or *PfΔp230p-2*, whereas a *p230p* transcript (259 bp) was amplified from WT gametocytes. We were able to amplify *p230* and *p48/45* transcripts (745 and 1219 bp respectively) from gametocytes of WT

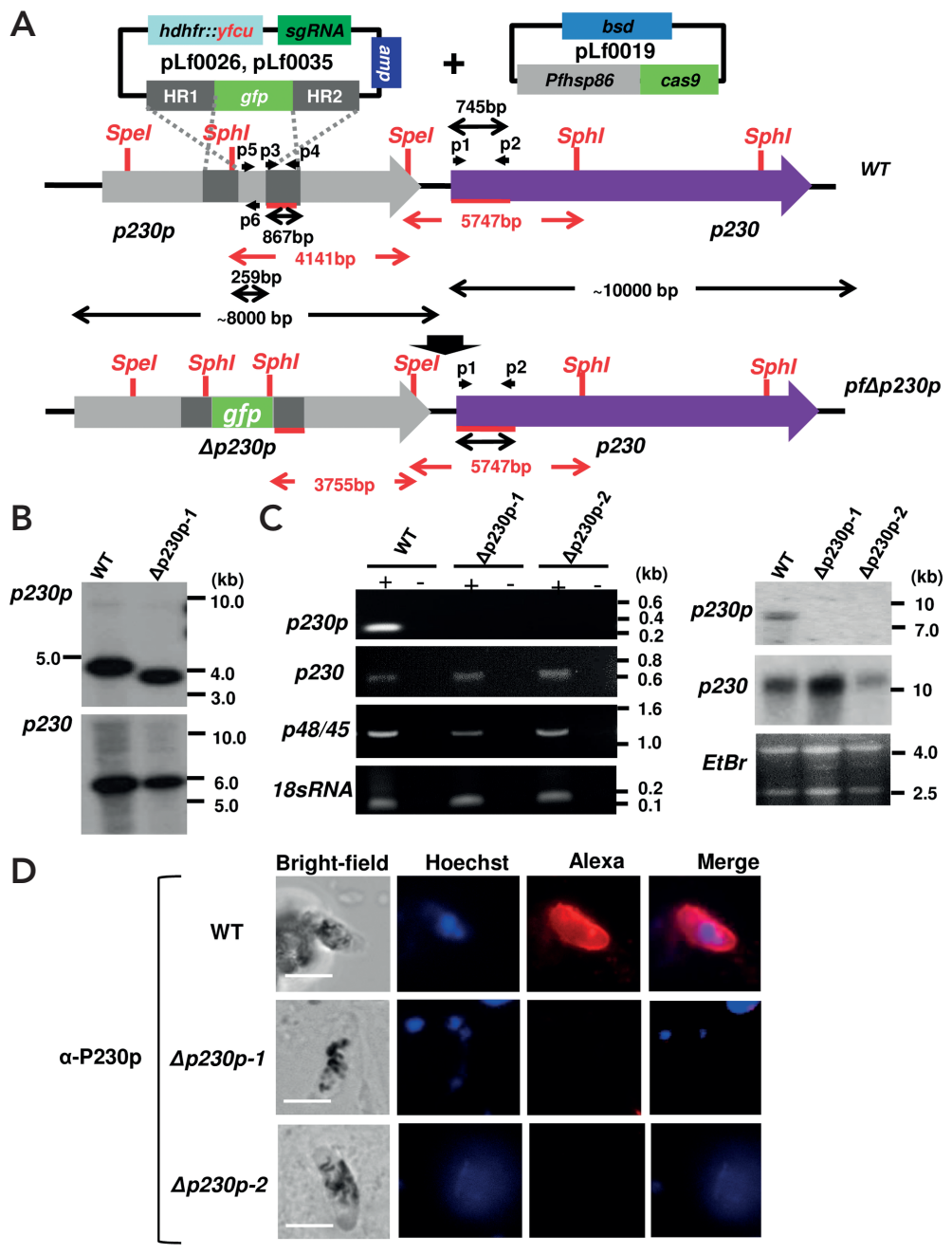

Figure 1. Generation and genotyping of *PfΔ230p* parasite lines and absence of *p230p* transcription in *PfΔp230p* parasites. **A**. Two *PfΔp230p* parasite lines were generated using CRISPR/Cas9 methodology as described previously [20]. The *p230p* gene was disrupted by insertion of a GFP-expression cassettes using plasmids pLf0026 (*cam* promoter driving GFP) or pLf0035 (*hsp70* promoter driving GFP). A schematic representation of the locus containing the paralogous genes *p230p* and *p230*, before and after insertion of the construct showing the location of the restriction sites (*Spe*I, *Sph*I),

sizes of restriction fragments (in red), location of primers (p) and the PCR amplicons and sizes of transcripts (in black) used to analyse correct disruption and transcription of the paralogous genes (**B, C**). HR1, HR2: *p230p* homology regions. The figure is not shown to scale. Primer sequences can be found in **Table S1**. **B**. Southern analysis of *Sph*I/*Spe*I restricted DNA of WT and *PfΔp230p-1* parasites confirms the specific and expected disruption of the *p230p* gene locus. DNA was hybridized with a probe targeting the homology region 2 (HR2; primers p3/p4) of *p230p* (left panel) and a specific probe of 745bp (primers p1/p2) of the 5'*p230* open reading frame (right panel). The hybridization pattern observed with first probe identified the expected different-sized DNA fragments in WT and *PfΔp230p-1* parasites (4141 bp and 3755 bp); the second probe hybridized to a single expected fragment (5747 bp) in both WT and *PfΔp230p-1,* indicating an unaltered *p230* locus. Uncropped images of the Southerns are shown in **Figure S3**. **C**. Transcription analysis of the 6-Cys family members *p230p, p230* and *p48/45* in WT and *PfΔ230p* parasites by RT-PCR and Northern blot. Left panel: RT-PCR amplified transcripts of *p230p* (primers p5/p6; expected size: 259bp), *p230* (primers p1/p2; expected size: 745bp), *p48/45* (primers p7/p8; expected size: 1219bp) and *18sRNA* (primers p9/p10; expected size: 165bp). + and – denote the presence or absence of reverse transcriptase. Uncropped images of gels are shown in **Figure S4**. Right panel: Northern blot analysis of *p230p* and *p230* transcripts confirming the absence of *p230p* and presence of *p230* transcripts in *PfΔ230p* parasites. Upper panel: hybridization with an internal probe (259bp) from *p230p* (primers p5/p6, WT expected size: ~8kb); middle panel hybridization with a probe against the 5'*p230* open reading frame (primers p1/p2, expected size: ~ 10kb); lower panel: ethidium bromide (*EtBr*) stained RNA as loading control. Uncropped images of the Northern blot analyses are shown in **Figure S5**. The size of expected RT-PCR products and transcripts are shown in black in **a**. Primer sequences are shown in **Table S1. D.** Immunofluorescence analyses of mature, stage V, WT and PfΔp230p gametocytes. Fixed cells were labelled with mouse anti-P230p polyclonal serum (anti-rMBP.PfB0400w) and with secondary conjugated antibodies anti-IgG Alexa Fluor® 594 (red). Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/gain times; anti-IgG Alexa Fluor® 594 (red). 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7µm.

and the *PfΔp230p* mutant lines. The lack of *p230p* transcripts and presence of *p230* transcripts in gametocytes of the mutant lines was confirmed by Northern blot analysis using the probes specific for the *p230p* and *p230* gene loci (**Figure 1C**).

In addition, we analysed P230p expression by immunofluorescence microscopy using polyclonal antiserum against P230p [17]. This antiserum reacted only to WT gametocytes and no signal was detected in *PfΔp230p* gametocytes (**Figure 1D**). Combined our analyses show correct disruption of *p230p* in *PfΔp230p* parasites resulting in absence of expression of *p230p* in gametocytes whereas the paralogous gene *p230* is transcribed. The staining pattern in WT gametocytes suggests P230p is located at the surface in the mature stage V gametocyte, and is in agreement with the localisation in *P. falciparum* gametocytes expressing a GFP-tagged version of P230p (P230p-GFP;[9]). We also analysed P230p expression in activated male gametocytes, both using the polyclonal antiserum against P230p and in gametocytes of the transgenic P230p-GFP line. We were unable to clearly detect P230p in male gametes using the polyclonal serum or in live gametes of the P230P-GFP line but, staining with anti-GFP antibodies demonstrated that the tagged-protein was present either in or on male gametes (**Figure S1A, B and C**). While P230p is expressed in/on male gametes it is likely to be weakly expressed given the low GFP fluorescence and that there was no detectable signal with polyclonal serum.

Mosquito transmission of PfΔp230p-1 and PfΔp230p-2 parasites is strongly reduced

Since multiple members of the 6-cys family play a role in mosquito transmission (i.e. P48/45, P47, P230; [5-7, 11-14]) we analysed the ability of mosquitoes to transmit the *PfΔp230p* lines. *A. stephensi* mosquitoes were fed with WT and *PfΔp230p* gametocytes using the standard membrane feeding assay and the number of oocysts and salivary gland sporozoites were determined at day 6 and day 14 respectively. We fed WT and *PfΔp230p* gametocytes in independent experiments and used different clones of the mutant parasites (WT, 7 experiments (exp.); *PfΔp230p-1* clone 0022cl1, 6 exp.; *PfΔp230p-1* clone 0022cl5, 5 exp.; *PfΔp230p-2* clone 0035cl4, 6 exp). In all experiments we observed a >98% reduction in oocyst development in mosquitoes that had been fed with *PfΔp230p* parasites; mean oocyst numbers ranging from 4 to 32 for WT-infected mosquitoes compared to 0.1 to 0.7 in *PfΔp230p*-infected mosquitoes (100 mosquitoes analysed per experiment; **Figure 2A, Table S3**). In *PfΔp230p*-infected mosquitoes we observed a maximum of 5 oocysts per mosquito compared to 40 after WT feeding . No sporozoites were observed in salivary glands of *PfΔp230p*-infected mosquitoes. These results indicate t2he *P. falciparum* P230p plays an important role in mosquito transmission.

The formation of ookinetes in PfΔp230p-1 and PfΔp230p-2 parasites is severely compromised

To better define the role of P230p in *P. falciparum* mosquito transmission we analysed gametocyte, gamete and ookinete formation of the two mutant lines. The *in vitro* production of male and female gametocytes of both *PfΔp230p-1 and PfΔp230p-2* were in the same range as WT parasites (**Table 1**). However, the number of 'retort-form' and mature ookinetes was reduced by >97% in midguts of *A. stephensi* mosquitoes at 22 h. after feeding (**Figure 2A**, **Table 1**). In *PfΔp230p* infected mosquitoes we observed no or very low numbers of retorts and mature ookinetes, with mean numbers ranging from only 0 to 1.5 (retorts) and 0 to 0.5 (mature ookinetes). In WT infected mosquitoes the numbers of retorts and mature ookinetes ranged between 6-21 and 7.5-27, respectively. This strong reduction in ookinetes numbers, indicate that either fertilisation or the development of fertilised female gametes is interrupted. We cannot discriminate between these two possibilities, since *P. falciparum* fertilised female gametes cannot easily be distinguished from unfertilized female gametes, thereby hampering quantification of fertilisation. However, since P230p is specifically expressed in male gametocytes the most likely explanation for the reduced ookinete formation is the inability of *PfΔp230p* male gametes to fertilize females. We therefore next analysed the formation of male gametes in more detail.

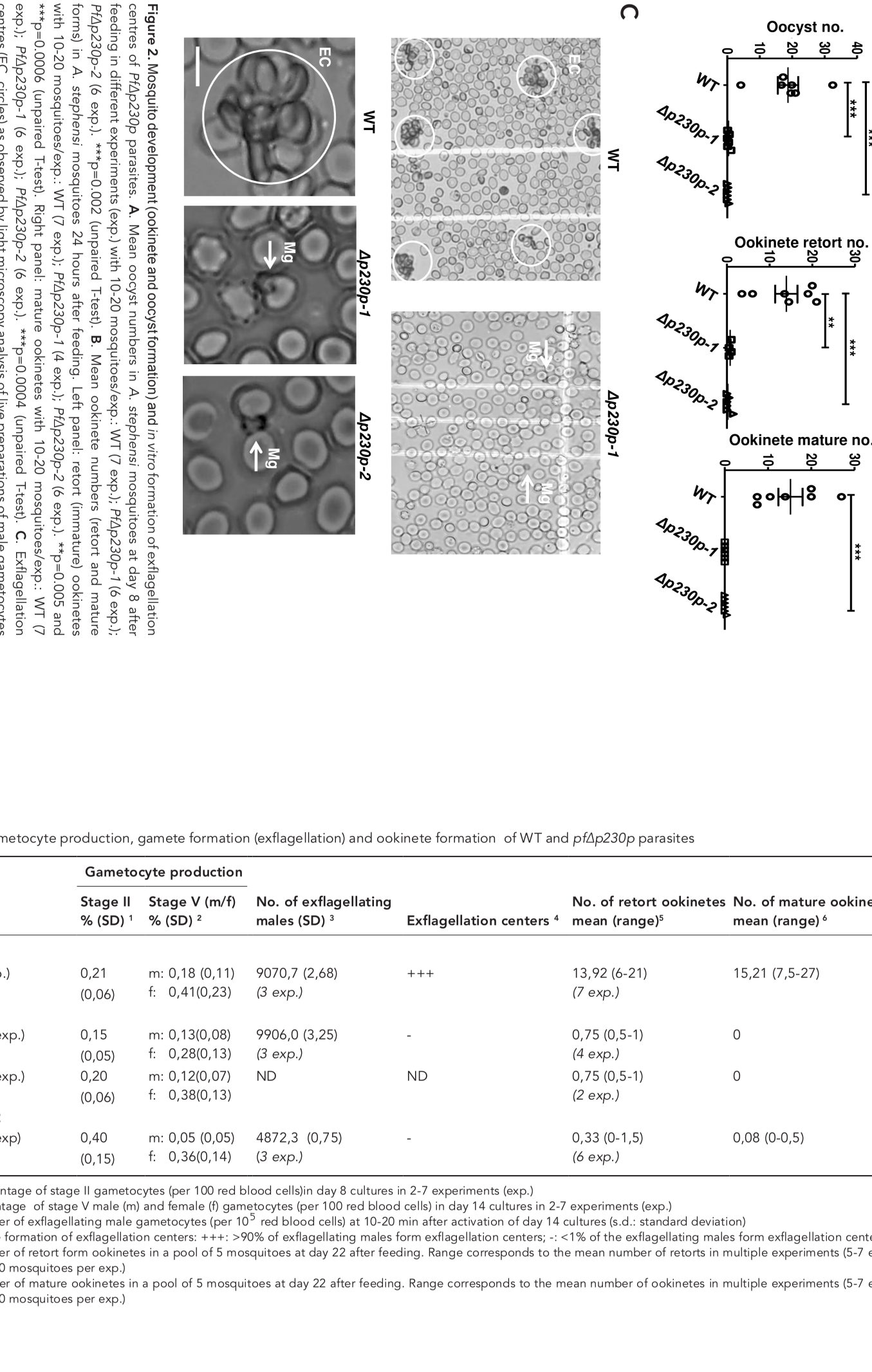

Figure 2. Mosquito development (ookinete and oocyst formation) and *in vitro* formation of exflagellation centres of *PfΔp230p* parasites. **A**. Mean oocyst numbers in *A. stephensi* mosquitoes at day 8 after feeding in different experiments (exp.) with 10-20 mosquitoes/exp.: WT (7 exp.); *PfΔp230p-1* (6 exp.); *PfΔp230p-2* (6 exp.). ***p=0.002 (unpaired T-test). **B**. Mean ookinete numbers (retort and mature forms) in *A. stephensi* mosquitoes 24 hours after feeding. Left panel: retort (immature) ookinetes with 10-20 mosquitoes/exp.: WT (7 exp.); *PfΔp230p-1* (4 exp.); *PfΔp230p-2* (6 exp.). **p=0.005 and ***p=0.0006 (unpaired T-test). Right panel: mature ookinetes with 10-20 mosquitoes/exp.: WT (7 exp.); *PfΔp230p-1* (6 exp.); *PfΔp230p-2* (6 exp.). ***p=0.0004 (unpaired T-test). **C**. Exflagellation centres (EC, circles) as observed by light microscopy analysis of live preparations of male gametocytes between 10 and 20 min after activation examined in a Bürker cell chamber. Only WT activated male gametocytes attach to red blood cells and form characteristic exflagellation centres (left panel; white circles). See also **Supplementary videos S1-6** for the absence/presence of exflagellation centres. Arrows indicate exflagellating male gametocytes (MG) of *PfΔp230p*. Scale bar, 7μm.

Table 1. Gametocyte production, gamete formation (exflagellation) and ookinete formation of WT and *pfΔp230p* parasites

Lines	Gametocyte production: Stage II % (SD) [1]	Gametocyte production: Stage V (m/f) % (SD) [2]	No. of exflagellating males (SD) [3]	Exflagellation centers [4]	No. of retort ookinetes mean (range)[5]	No. of mature ookinetes mean (range) [6]
WT						
NF54 (7 exp.)	0,21 (0,06)	m: 0,18 (0,11) f: 0,41(0,23)	9070,7 (2,68) *(3 exp.)*	+++	13,92 (6-21) *(7 exp.)*	15,21 (7,5-27)
ΔPfs230p-1						
0022cl1 (4 exp.)	0,15 (0,05)	m: 0,13(0,08) f: 0,28(0,13)	9906,0 (3,25) *(3 exp.)*	-	0,75 (0,5-1) *(4 exp.)*	0
0022cl5 (2 exp.)	0,20 (0,06)	m: 0,12(0,07) f: 0,38(0,13)	ND	ND	0,75 (0,5-1) *(2 exp.)*	0
ΔPfs230p-2						
0035cl4 (6 exp)	0,40 (0,15)	m: 0,05 (0,05) f: 0,36(0,14)	4872,3 (0,75) *(3 exp.)*	-	0,33 (0-1,5) *(6 exp.)*	0,08 (0-0,5)

[1] Mean percentage of stage II gametocytes (per 100 red blood cells)in day 8 cultures in 2-7 experiments (exp.)
[2]Mean percentage of stage V male (m) and female (f) gametocytes (per 100 red blood cells) in day 14 cultures in 2-7 experiments (exp.)
[3] Mean number of exflagellating male gametocytes (per 10^5 red blood cells) at 10-20 min after activation of day 14 cultures (s.d.: standard deviation)
[4] Level of the formation of exflagellation centers: +++: >90% of exflagellating males form exflagellation centers; -: <1% of the exflagellating males form exflagellation centers
[5] Mean number of retort form ookinetes in a pool of 5 mosquitoes at day 22 after feeding. Range corresponds to the mean number of retorts in multiple experiments (5-7 exp. per line; 10-20 mosquitoes per exp.)
[6] Mean number of mature ookinetes in a pool of 5 mosquitoes at day 22 after feeding. Range corresponds to the mean number of ookinetes in multiple experiments (5-7 exp. per line; 10-20 mosquitoes per exp.)

PfΔp230p-1 and PfΔp230p-2 male gametes are unable to generate exflagellation centres

Both gametocyte production and sex ratio of mature gametocytes at day 14 were comparable between WT and *PfΔp230p* parasites (**Table 1**) and morphologically, at the light microscopy level, there is no difference between WT and *PfΔp230p* gametocytes (data not shown). After activation in FCS mature gametocytes from both WT and *PfΔp230p* cultures readily formed high numbers of exflagellating male gametocytes as observed by light-microscopy. We estimate that >90% of stage V male *PfΔp230p* gametocytes showed exflagellation (from 3 experiments) (**Table 1**). These observations indicate that the formation of male gametes is not affected by the absence of P230p. However, a striking difference was the absence of *PfΔp230p* male gamete attachment to uninfected RBC and the formation of exflagellation centres observed 15-30 min post activation (**Figure 2B and Supplementary videos S1-S6**). Such exflagellation centres generally consist of one or more exflagellating male gametes attaching to a number of RBC [21]. While WT stage V gametocytes formed such centres by more than 90% of the activated male gametocytes, this was observed in less than 5% of activated *PfΔp230p* males (**Table 1**). These results indicate that in the absence of P230p, male gametes were incapable of effectively attaching to RBC. This phenotype is very similar to the phenotype described for *P. falciparum* mutants lacking P230 and indicates that both P230 and P230p play a role in interactions of male gametes with RBC. Whether the inability of male gametes to interact with RBC is solely responsible for the reduced ookinete formation, or whether P230p has an additional role in fertilisation, is unknown. It has been shown that the 6-cys family members, P230 and P48/45, form complexes with other proteins on the surface of gametes [22, 23]. We therefore examined if the expression of P230 and P48/45 was altered in activated *PfΔp230p-1 and PfΔp230p-2* gametocytes by immunofluorescence analysis. Using anti-P230 and P48/45 antibodies we demonstrated that P230 and P48/45 were present in both activated *PfΔp230p* female and male gametocytes/gametes where staining patterns were comparable to what was observed in activated WT gametocytes (**Figure 3A, B and Figure S1D**). The combined results indicate that the formation of exflagellation centres and subsequent reduced ookinete formation is directly P230p dependent and not a consequence of the loss of P230 or P48/45 on gametes in the *PfΔp230p* gametes.

The role of P230 and P230p in *P. falciparum* gamete binding to RBC is different to the role of these proteins in the rodent parasite *P. berghei*. Single gene-deletion mutants lacking expression of either P230 or P230p in *P. berghei* exhibit formation of exflagellation centres like WT, indicating that male gametes of these mutants bind normally to RBC [5]. To examine a possible compensatory role in RBC binding of the two *P. berghei* paralogs we generated a *p230* and *p230p* double gene deletion mutant (**Figure S2A and B**). Activated male gametocytes of this mutant, *PbΔp230Δp230p*, formed WT-like levels of exflagellation centres (**Figure S2C, Supplementary videos S7-12, Table S4**) demonstrating an absence of a role of these proteins in RBC binding of *P. berghei* male gametes.

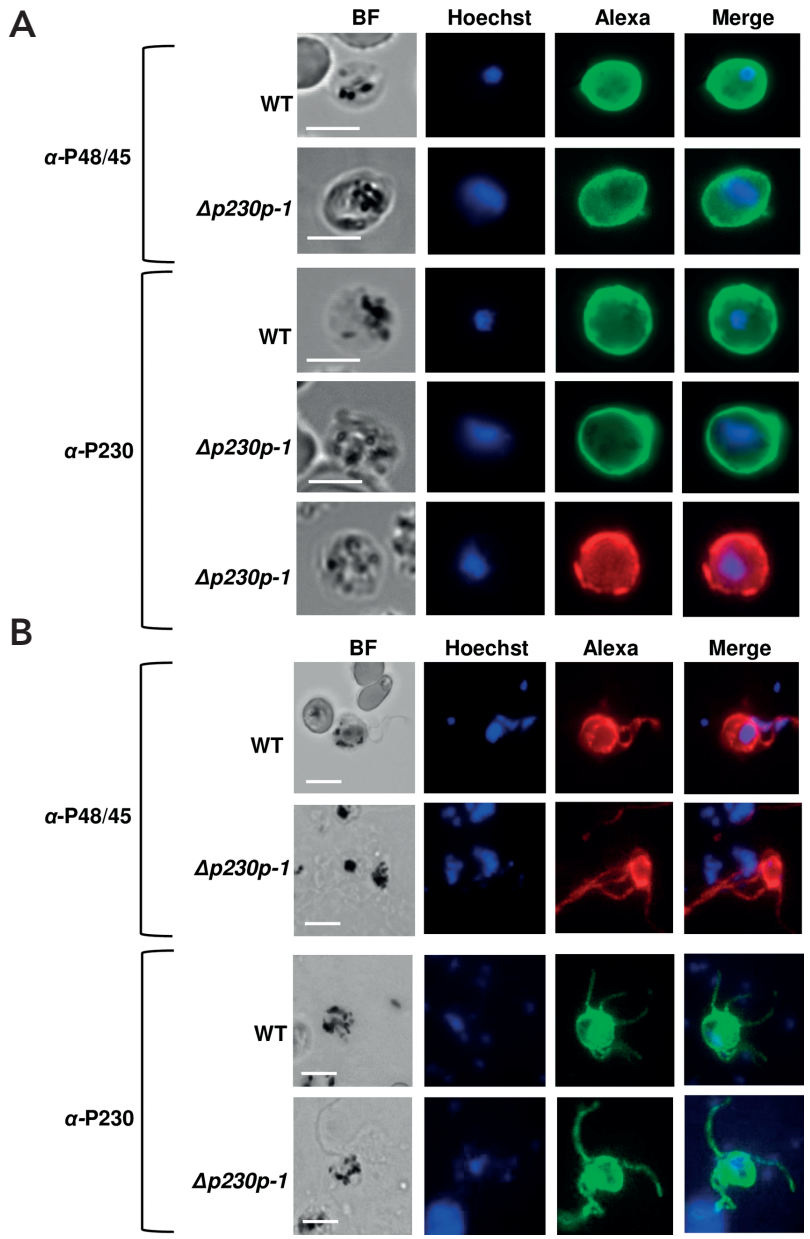

Figure 3. Expression of P230 an P48/45 in activated female and male gametes of *PfΔp230p-1*. **A**. Immunofluorescence analyses of female gametes 30 minutes after gametocyte activation in fetal calf serum. Unfixed parasites were labelled with mouse anti-P230 (MAb 63F2A2) or rat anti-P48/45 (MAb 85RF45.1) antibodies followed by secondary conjugated antibodies (i.e. anti-rat, anti-mouse IgG Alexa Fluor® 488 (green) or anti- mouse IgG Alexa Fluor ® 594 (red)). Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor® 488 (green) 0.7 s; anti-IgG Alexa Fluor ® 594 (red) 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7µm. **B.** Immunofluorescence analyses of male gametes 15 minutes after gametocyte activation in fetal calf serum. Cells were fixed with methanol and labelled with

- mouse anti-P230 (MAb 63F2A2) or rat anti-P48/45 (MAb 85RF45.1) antibodies followed by secondary conjugated antibodies (i.e. anti-mouse IgG Alexa Fluor® 488 (green) or anti-rat IgG Alexa Fluor® 594 (red)). Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor ® 488 (green) 0.7 s; anti-IgG Alexa Fluor ® 594 (red). 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7µm.

Finally, we examined fertility of female gametes of the *PfΔp230p* lines, by crossing the GFP-expressing *PfΔp230p* gametocytes with WT gametocytes and examining parasite development in mosquitoes. Mosquitoes with both GFP-positive and GFP-negative oocysts were obtained in multiple experiments (**Figure 4; Table 2)**. GFP-positive oocysts can only result from cross-fertilisation of WT gametes and *PfΔp230p* gametes. In view of the male-specific expression of P230p and the male phenotype of *PfΔp230p* parasites, the presence of the GFP-positive oocysts most likely result from cross-fertilisation between WT male gametes and *PfΔp230p* female gametes. These observations are in support of normal fertility of *PfΔp230p* female gametes and reduced fertility of *PfΔp230p* male gametes.

Discussion

We demonstrate that *P. falciparum* P230p plays a vital role in parasite transmission through mosquitoes. Mutants lacking expression of P230p (*PfΔp230p*) have a strong reduction (>98%) in ookinete formation, which in turn results in a strong reduction in oocyst formation and absence of sporozoites in salivary glands. We show that the *PfΔp230p* male gametes have lost the capacity to bind to RBC and could not form the characteristic exflagellation centres. A function of P230p in male gamete fertility is in agreement with male specific expression of *Pf*P230p and concomitant absence in female gametocytes/gametes [8-10, 17-19]. Indeed the results of crossing experiments in mosquitoes, performed using a mixture of WT and *PfΔp230p* gametocytes, indicate that *PfΔp230p* females retain their fertility. The important role that *P. falciparum* P230p plays in mosquito transmission does not match the redundant function of P230p in the rodent parasites *P. berghei* and *P. yoelii*. Rodent parasites also express P230p specifically in male gametocytes [24], but mutants lacking P230p have no discernible defect and exhibit normal gametocyte/gamete formation, are fully able to form exflagellation centres and mosquito transmission is similar to WT parasites [5, 15]. We also demonstrate that the *P. berghei* P230 is not compensating for the loss of its paralogue P230p, since activated male gametocytes of *P. berghei* mutants lacking expression of both P230 and P230p can still bind to RBCs and form exflagellation centres. These observations demonstrate a critical difference in the function P230p performs in rodent and human malaria parasites. For a few other 6-Cys proteins there has been evidence for functional differences between the orthologs of rodent and human parasites. For example, the female-specific P47 protein is vital for the fertility of *P. berghei* female gametes, while this protein appears not to be crucial for *P. falciparum* female gamete fertility [5, 11]. Analysis of *P. berghei* mutants lacking P45/48 and

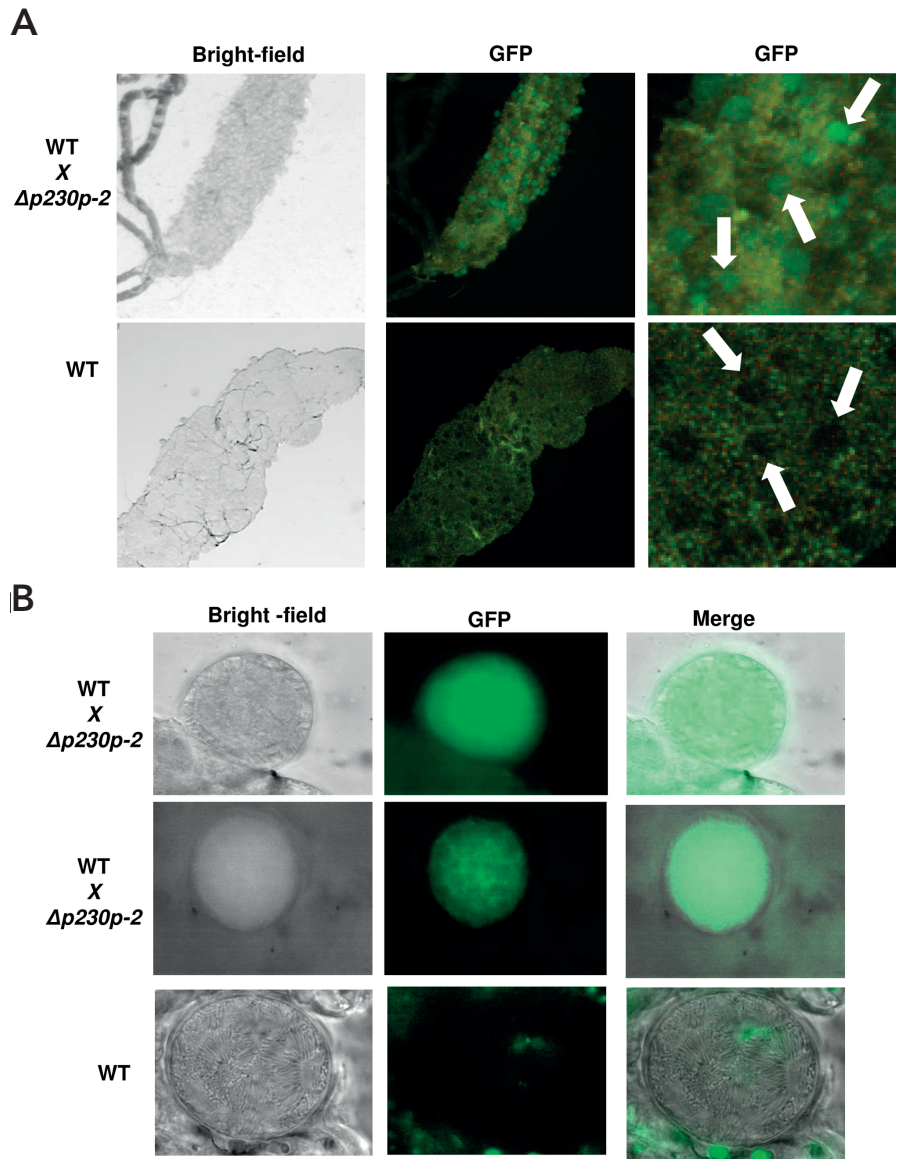

Figure 4. Crossing of GFP-expressing *PfΔp230p* gametocytes with WT gametocytes results in the formation of GFP-positive oocysts. **a.** GFP-positive oocyst in midguts of *A. stephensi* mosquitoes fed on a mixture of *PfΔp230p-2* and *WT* gametocytes (day 10 after feeding). Arrows indicate GFP-positive oocysts in the WT and *PfΔp230p-2* cross and GFP-negative oocysts in WT fed mosquitoes.**b.** GFP-positive and GFP-negative oocysts in mosquitoes fed on a mixture of *PfΔp230p-2* and WT gametocytes or only WT gametocytes (day 10 after feeding). **See Table 25** for the ratio of GFP-positive and GFP-negative oocysts in mosquitoes fed on a mixture of *PfΔp230p-2* and *WT* gametocytes. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (GFP 0.7 s; bright field 0.62 s (1x gain)).

Table 2. GFP-positive and GFP-negative oocysts after crossing P. falciparum WT and *pfΔp230p* gametocytes.

Parasites	Gametocyte production Stage V (m/f)%(SD)[a]	No. of exflagellating males[b]	Ratio WT/ *Δp230p* gams in cross[c]	No. of oocyst Mean (range)[d]	GFP positive oocyst (%)[e]
Cross A:					
WT	m: 0,5 (0,5) f: 1,3 (1,2)	0,5	**1/1 (1 exp.)**	**13 (1 exp.)**	0
Δp230p-2	m: 0,1 (0,4) f: 0,2 (1,2)	0,1			
Cross B:					
WT	m: 0,5 (0,8) f: 1,0 (0,8)	0,5	**1/2 (2 exp.)**	**40 (50-60) (2 exp.)**	58%
Δp230p-2	m: 0,9 (0,6) f: 1,8 (0,8)	0,8			
Cross C:					
WT	m: 0,5 (0,8) f: 1,0 (0,8)	0,2	**1/3 (1 exp.)**	**59 (1 exp.)**	41%
Δp230p-2	m: 0,6 (0,7) f: 1,1 (0,4)	0,3			

a. Mean percentage of stage V male (m) and female (f) gametocytes (per 100 red blood cells) in day 14 cultures in 1-2 experiments (exp.). SD: Standard deviation.
b. Mean number of exflagellating male gametocytes (per mL of culture) at 10-20 min after activation of day 14 gametocyte cultures (SD: standard deviation)
c. WT and *PfΔp230p* gametocytes were mixed in different ratios (1:1, 1:2, and 1:3) based on exflagellating male gametocytes counts per ml of gametocyte culture after activation with FCS.
d. Mean number of oocysts per mosquito at day 8 after feeding. Range corresponds to the mean number of oocyst in different experiments (1-2 exp. per crossing; 10-30 mosquitoes per exp.)
e. Percentage of GFP positive oocyst analysed in 5 individuals mosquitoes (1-2 exp.)

P230 demonstrates that these proteins are male-specific fertility factors [5, 6]. In contrast, *P. falciparum* P48/45 and P230 are expressed in both males and female gametes [8-10], which may suggest a role for these proteins in both male and female gamete fertility. Also other proteins expressed in gametocytes/gametes functional differences have been demonstrated between the equivalent proteins in rodent and human malaria parasites, for example members of the LCCL protein family. In rodent parasites most LCCL members are expressed after fertilisation, in the ookinete stage, and play a role in sporozoite formation [25, 26], whereas in *P. falciparum* these proteins are expressed in gametocytes and are part of protein complexes on the surface of gametocytes/gametes [27, 28].

The lack of RBC binding of *PfΔp230p* male gametes is similar to the phenotype of *P. falciparum* mutants lacking expression of its paralog, P230 [7]. These observations suggest that both proteins have a similar, but not interchangeable, function in RBC binding. We provide evidence that *PfΔp230p* male and female gametes retain expression of both P230 and P48/45, indicating that the lack of RBC binding is not due to the absence of

expression of P230 and/or P48/45. These observations would suggest that RBC binding of male gametes is not due to direct interactions of P230 to RBC receptors as was also demonstrated in the studies using males lacking expression of P230 [7]. Moreover, P230 unlike P230p is also expressed at the surface of female gametes [8-10, 23], which makes it less likely that P230 interacts directly with RBC.

P230 and P48 form complexes with several other proteins at the surface of female gametes and zygotes [23, 29, 30]. Given that of P48/45, P230 and P230p are expressed in male gametes, it is conceivable that comparable complexes that may include additional proteins, are also formed at the surface of male gametes. The absence of either P230 or P230p may affect the correct formation of such protein complexes at the gamete surface, which may in turn lead to the same loss of RBC binding phenotype observed in mutants that lack either paralog. This would indicate that neither P230 nor P230p but rather other parasite proteins/factors are directly responsible for binding to RBC receptors. Future studies are needed to unravel in more detail the molecular interactions between male gametes and RBC and the *Plasmodium* ligand(s) that bind to the putative proteins, sialic acid and/or glycophorin receptors on the RBC surface [21].

Whether the reduction in fertilisation and ookinete formation within the mosquito midgut of parasites lacking either P230 or P230p is directly due to the inability of male gametes to bind to RBC or whether these proteins have an additional role in fertilisation remains unknown. Studies on male gametes lacking P230 showed that the inability to form exflagellation centres did not affect the release of male gametes from activated gametocytes [7] and we also observed *in vitro* that *PfΔp230p* male gametes were released after gametocyte activation. It has been suggested that the RBC interactions may trigger changes in the gamete that are required for fertilisation such as the release of additional proteins, which through a process analogous to sperm capacitation, permit the male gamete to be able to bind to molecules in the zona pellucida of the oocyte and thereby initiating the process of male penetration of the female gamete [21]. Unfortunately, efficient *in vitro* assays for *P. falciparum* fertilisation are absent [31] and fertilised female gametes cannot easily be distinguished from unfertilized female gametes, thereby hampering more detailed analyses and quantification of fertilisation events.

Our study expands the role of the 6-Cys proteins in fertilisation and specifically demonstrates that P230p, like P230 and P48/45, has a clear and vital role in *P. falciparum* male fertility, zygote formation and parasite transmission through mosquitoes.

MATERIALS AND METHODS

Parasites and culture

We analysed wild type (WT) *P. falciparum* NF54 parasites and two mutant lines *PfΔp230p-1* (GFP@cam clones 0022cl1 and 0022cl5) and *PfΔp230p-2* (GFP@hsp70 clone 0035cl4) with a disrupted *p230p* gene locus (PF3D7_0208900). These mutants were generated in NF54 parasites by introducing a GFP-reporter cassette into the *p230p* gene locus using

CRISPR/Cas9 methodology [20]. In the two mutants, GFP is either under the control of the promoter from *calmodulin* (*cam*; PF3D7_1434200) or from *heat shock protein 70* (*hsp70*; PF3D7_0818900). The genotype and phenotype of (asexual) blood stages of these mutants have been reported previously [20]. WT NF54 parasites [32] had been obtained from the Radboud University Medical Center (Nijmegen, The Netherlands). NF54 parasites were cultured following the standard conditions in RPMI-1640 culture medium supplemented with L-Glutamine 25mM HEPES (Gibco Life Technologies) and 50 mg/L hypoxanthine (Sigma). Culture medium was supplemented with 10% human serum and 0.225% $NaHCO_3$. Parasites were cultured at a 5% hematocrit under 4% O_2, 3% CO_2 and 93% N_2 gas-conditions at 75 rpm at 37°C in a semi-automated culture system [33]. Fresh human serum and human red blood cells (RBC) were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria research and microbiology safety-tests).

In addition, a *P. falciparum* (3D7) transgenic line that expresses a GFP-tagged version of *Pfp230p* (*Pfp230p*-GFP) was analysed for P230p expression and localisation. This line has been engineered to express endogenous PfP230p fused to GFP to its carboxyl terminal and was generated using a single cross-over recombination strategy [9].

Two different *P. berghei* ANKA mutants were analysed that have been previously generated. One with a *p230p* gene disruption (line 676m1cl1; *PbΔp230p*; RMgm-29; www.p*berghei*.eu) [34] and the other with a *p230* gene disruption (line 310cl1; *PbΔp230*; RMgm-350; www.p*berghei*.eu) [5]. In addition, we generated a double gene deletion *P. berghei* ANKA mutant with both the *p230p* (PBANKA_0306000) and *p230* (PBANKA_0306100) gene loci disrupted (see below).

Animal ethics statement

Female OF1 mice (6–8 weeks old; Charles River/Janvier) were used. All animal experiments of this study were in accordance with relevant guidelines and regulations approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

Generation of the P. berghei double knock-out mutant PbΔp230Δp230p

To generate a *P. berghei* mutant lacking expression of both P230 and P230p we disrupted the *p230* locus in the existing *PbΔp230p* mutant (676m1cl1; see above) which has a disrupted *p230p* locus. To disrupt *p230* we used a DNA construct that had been used to create the mutant *PbΔp230* (310cl1; see above). This construct (pL1139) integrates by double cross integration and replaces (part of) the *p230* locus with the selectable marker cassette containing *Toxoplasma gondii* dihydrofolate reductase/thimidylate synthase (*tgdhfr/ts*) [5]. Parasites of line 676cl1 were transfected with this construct (exp. 2764) using standard transfection technologies and selection with pyrimethamine [35]. Selected

parasites were cloned by limiting dilution and mutant 2764cl3 was used for genotype and phenotype analysis.

Genotyping and phenotyping P. berghei mutant PbΔp230Δp230p

Correct disruption of the *p230p* and *p230* gene loci was performed by diagnostic PCR-analysis and Southern analysis of pulsed field gel (PFG) separated chromosomes as described previously [35]. Briefly, for the PCR-analysis confirmation of disruption of *p230* was performed using the primers p13/p14 for 5′ integration and p17/p18 for 3′ integration of the construct and for *p230p*, 5′ and 3′ integration with primer pairs p21/p22 and p25/p26, respectively (see **Figure S2** and **Table S1** for details of the primers and the PCR fragments). For Southern analysis, diagnostic probes against the 3′UTR of *pbdhfr/ts* and the *tgdhfr/ts* selectable marker were used.

In vitro activation of gametocytes to determine exflagellation, formation of exflagellation centres and formation of ookinetes were performed as described [5, 35]. In brief, 10-20 µl of tail blood from infected mice containing gametocytes was diluted in 1 ml of activation medium. Within 12-20 min after activation, exflagellating male gametocytes and exflagellation centres were quantified in a Bürker cell counter and 18-24 h later the number of zygotes/ookinetes formed was quantified.

Genotyping P. falciparum mutants PfΔp230p-1 and PfΔp230p-2

Diagnostic PCR and Southern analysis of restricted genomic DNA to confirm disruption of *p230p* in mutants *PfΔp230p-1* and *PfΔp230p-2* have been reported [20]. We performed additional Southern analysis to confirm disruption of *p230p* and to confirm that the neighbouring *p230* gene locus (PF3D7_0209000) remained unmodified. Total DNA was isolated from infected red blood cells (iRBC) obtained from 10ml cultures (parasitemia 5-10%, 5% hematocrit), pelleted by centrifugation (400 *g*; 5 min). RBC were lysed with 5-10 ml of cold (4°C) erythrocyte lysis buffer (10x stock solution 1.5 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M Na_2EDTA; pH 7.4; [35] and parasites pelleted by centrifugation (400*g* during 5 min) and treated with RNAse and proteinase-K before DNA isolation by standard phenol-chloroform methods. Genomic DNA was digested with *Spe*I and *Sph*I restriction enzymes (4 h at 37°C) to confirm the specific disruption of *Pfp230p* locus. Restricted DNA was hybridized with 2 probes: one targeting the *p230p* homology region 2 (HR2) and one targeting the 5′ *p230* open reading frame (*5′-p230*) amplified from WT NF54 genomic DNA by PCR using the primers P3/P4 for HR2 and P1/P2 for *5′-p230*, respectively (see **Table S1** for details of the primers).

Transcriptional analyses of 6-cys family proteins in the mutants PfΔp230p-1 and PfΔp230p-2

To analyse transcription of 6-cys family proteins *P. falciparum* gametocytes were generated using standard culture conditions (see above) with some modifications [33]. Briefly, parasites from asexual stage cultures were diluted to a final parasitemia of 0.5% and

cultures were followed during 14 days without refreshing RBC. After 9 days these cultures were treated with 50mM of N-acetyl-D-glucosamine (Sigma) to kill asexual stages and to enrich for gametocytes. At day 14 the cultures were harvested and infected RBC (iRBC), enriched for gametocytes, pelleted by centrifugation (400*g* during 5min), washed three times with 1X PBS and the iRBC lysed with saponin following standard procedures [36]. Total RNA was isolated from the pelleted parasites using the Kit RNA Pure Link ™ RNA Mini kit (Invitrogen) according to the manufactures instructions. Northern blot analysis on the isolated RNA, was performed as previously described [36] using probes amplified from genomic DNA from WT NF54 parasites; one targeting an internal fragment (259bp) of *p230p* with primers P5/P6 and the other targeting a fragment (754bp) of the 5′ *p230* open reading frame with primers P1/P2 (see **Table S1** for details of the primers). RNA (1-5µg) isolated from the iRBC was further purified for RT-PCR analysis by adding 1X DNase I digestion buffer (Promega), 20 U of RNase inhibitor (RNasin, Promega) and 20 U of DNase I (Promega); this was incubated for 45 min at 37°C followed by chloroform/isoamyl alcohol purification and RNA precipitated in absolute ethanol[36] Subsequently, RT-PCR was performed using standard methods [36]. Briefly, 1-3µg of RNA was collected for first strand cDNA synthesis using the kit SuperScript III (Invitrogen) and PCR amplification (annealing temperatures ranging 50-57°C) was performed with KOD polymerase (Invitrogen). For amplification of the *Pf48/45* gene (PF3D7_1346700) the primers P7/P8 were used, for *Pfp230* primers P1/P2, for *Pfp230p* primers P5/P6 and for *18S rRNA* primers P9/P10 (see **Table S1** for details of the primers).

Expression analysis of 6-cys family proteins in P. falciparum gametocyes by immunofluorescence assay (IFA)

To analyse the expression of *Pf*P48/45 and *Pf*P230 in live gametocytes by immunofluorescence microscopy, 500µl of the gametocyte culture was pelleted (400 *g* 30 s) and gametocytes activated in 1 ml of fetal calf serum (FCS) for 1h at room temperature and samples collected for live fluorescence microscopy. To analyse *Pf*P48/45 and *Pf*P230 expression in fixed (male) gametocytes, 20 µl of the activated cells were collected 15-20 min after activation. This gamete enriched solution was placed on a microscope slide, dried for 10 min, and fixed with ice-cold methanol for 5 min. After fixation the slides were blocked with 10% of FCS in 1X PBS for 1h. Live and fixed cells were washed with 1X PBS and incubated with monoclonal antibodies against *Pf*P48/45 (rat MAb 85RF45.1; 1:200 dilution of 5µg/ml stock solution [37]), PfP230 (mouse MAb 63F2A2; 1:200 dilution of 5µg/ml stock solution [38]) for 30 min at 4°C for live imaging and 1h at room temperature for fixed slides. Subsequently, cells were rinsed 3 times with 1X PBS and incubated with the secondary antibodies Alexa FLuor®488/594-conjugated chicken anti-rat and anti-mouse (Invitrogen Detection technologies), respectively (both at 1:200). Finally, the cells were stained with the DNA-specific dye Hoechst-33342 at a final concentration of 10µM. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and a coverslip placed

onto the cells and sealed with nail polish. Stained cells (live and fixed) were analysed for fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa: 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain). To analyse *Pf*P230p expression in stage V gametocytes by immunofluorescence slides for microscopy analysis were prepared as follows: 20 µl of the cell suspension containing activated gametocytes was placed per well of a 8-well black cell-line diagnostic microscope slide (Thermo Scientific), that was air dried, fixed with ice-cold absolute methanol (2 min) and subsequently washed 3 times with 1X PBS. Cells were permeabilized with 0.5% of Triton X-100 in 1X PBS for 1h and blocked with 10% FCS in 1X PBS. 20 µl of polyclonal serum raised in mice against recombinant *Pf*P230p (1:200; anti-rMBP.PfB0400w 1:200 dilution [17]) was incubated with the fixed gametocytes for 1 h at room temperature and slides were washed 3 times with 1XPBS. Subsequently each well was incubated with 20 µl goat-anti-mouse secondary IgG monoclonal antibody conjugated to Alexa FLuor®594-(Supplier; 1:200 dilution) for 1h at room temperature. Slides were then washed 3 times with 1X PBS and stained with 20 µl of Hoechst-33342 in 1XPBS (10 µM) for 30 min at 37 °C. The slides were washed 3 times in 1X PBS and the cells were analysed for fluorescence using a Leica fluorescence MDR microscope (see above for details).

Further analysis of *Pf*P230p expression was performed by detecting the GFP pattern in the live or fixed gametocytes of *Pfp230p*-GFP line. Rabbit anti-GFP IgG (Invitrogen; 4 µg/ml) and Goat anti-rabbit IgG conjugated to Alexa FLuor®488 (Invitrogen; 4 µg/ml) were used for detection of GFP in fixed activated mature gametocytes.

Phenotype analysis of gametocytes/gametes and mosquito stages of mutants PfΔp230p-1 and PfΔp230p-2

Gametocyte development was analysed in gametocyte cultures, established as described above. Exflagellation was determined after activation of *P. falciparum* stage V gametocytes with FCS. To activate gametocytes 20 µl of the gametocyte cultures at day 14 were diluted 1:1 with FCS at room temperature. Gametes and exflagellation centres were examined and quantified 10-20 min after activation using a Bürker cell counter.

The number of male gametocytes per 10^5 red blood cells (RBC) was determined in stage V gametocyte cultures by analysing Giemsa stained slides. Quantification of exflagellating males of these cultures was performed in triplicate, using a Bürker chamber (at 40X magnification). The number of exflagellating males is given as the number of exflagellating males observed per $1x10^5$ of total red blood cells (RBC).

Exflagellation center formation was determined by counting the number exflagellating males adhering to multiple red blood cells and forming characteristic dense clusters of RBC. Exflagellating males, which did not adhere to RBC and failed to form a characteristic dense cluster of RBC were scored as 'non-adhering' males. +++ denotes that more than 90% of the exflagellating males formed the dense RBC clusters and – denotes that less

than 1% of exflagellating males formed the dense RBC clusters. For analysis of mosquito stages (ookinetes, oocysts and sporozoites) *A. stephensi* were infected using the standard membrane feeding assay (SMFA) [39, 40]. Ookinetes were analysed and counted 22 h after feeding. Oocyst and salivary gland sporozoites were counted at day 6 and day 14 post feeding, respectively. For counting sporozoites, salivary glands from 10 mosquitoes were dissected and homogenized in a homemade glass grinder in 100 µl of RPMI-1640 (pH 7.2) and sporozoites were analysed in a Bürker cell counter using phase-contrast microscopy.

Cross-fertilisation of WT and *PfΔp230p* gametocytes was performed by mixing gametocytes obtained from enriched gametocyte cultures (see above) from WT and *PfΔp230p* and feeding these mixtures to mosquitoes using SMFA. In different experiments WT and *PfΔp230p* gametocytes were mixed in different ratios (1:1, 1:2, and 1:3) based on exflagellating male gametocytes counts per ml of gametocyte culture after activation with FCS. At day 10 after feeding oocyst development was analysed with a fluorescence stereomicroscope Leica MZ16 FA and GFP-fluorescence was visualized using GFP filter settings (GFP exposure time: 4,2 s). Pictures were recorded using a DM2500 digital camera.

Statistics

All data were analyzed using the GraphPad Prism software package 5.04 (GraphPad Software, Inc). To calculate significant levels for ookinete and oocyst numbers the unpaired Student's *t*-test was used.

Acknowledgements

C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). A.S. Othman Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). Jun Miao and Liwang Cui were partially supported by grant (R01AI104946) from National Institute of Allergy and Infectious Diseases, NIH.

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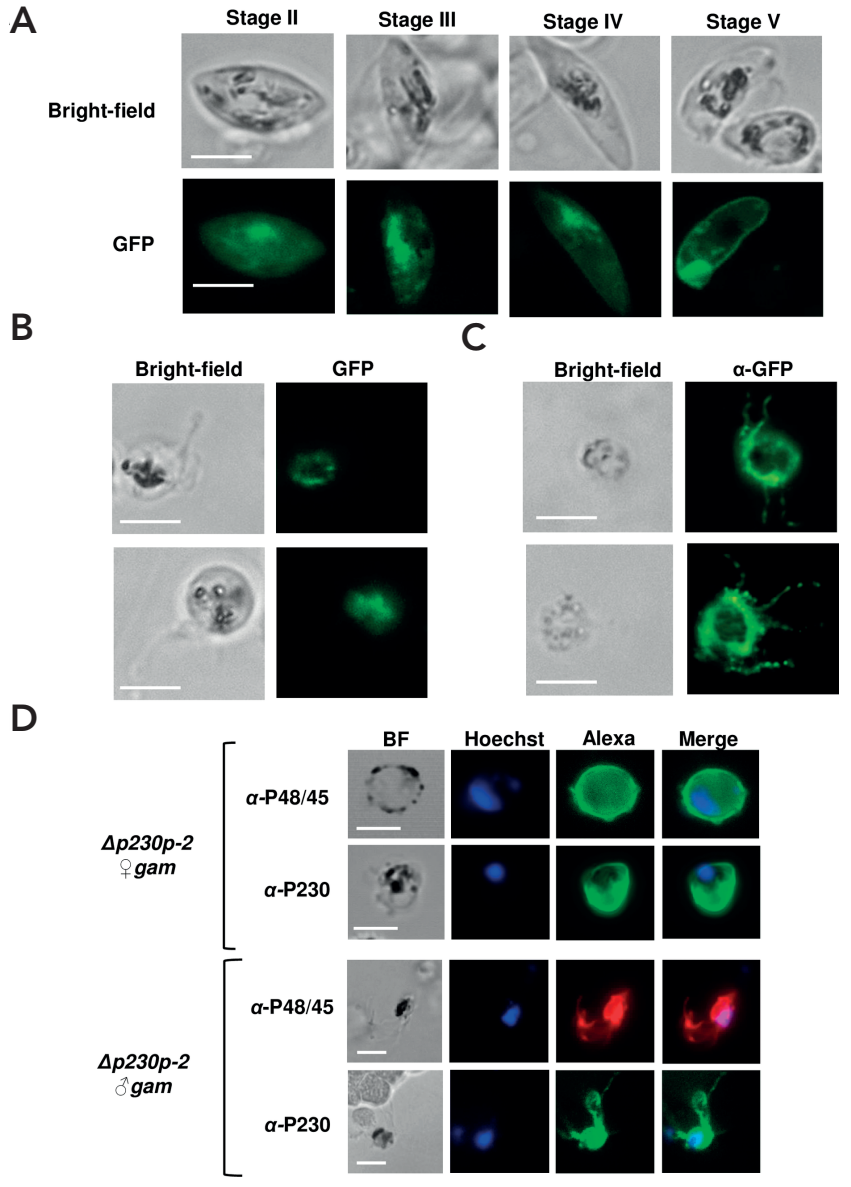
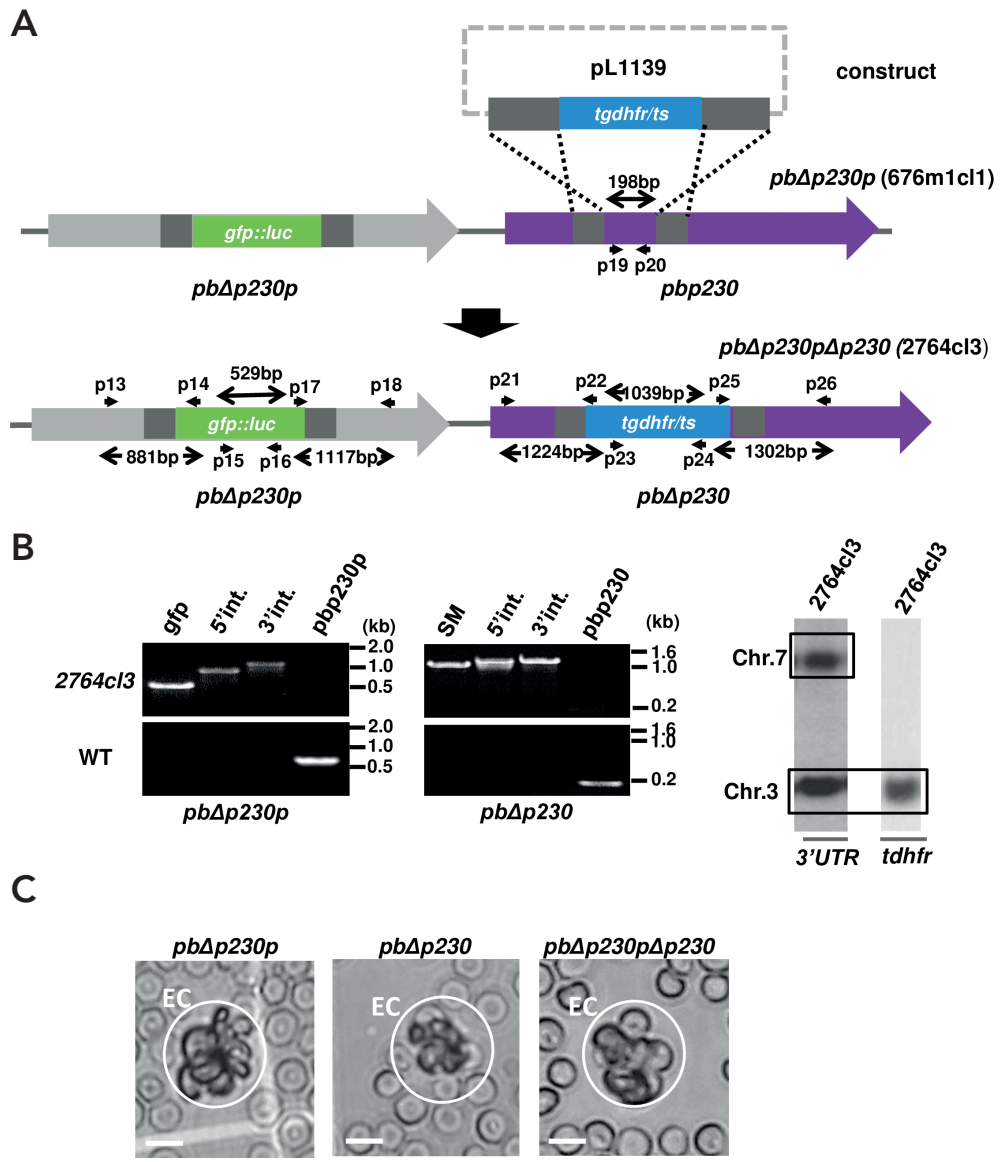
Supplementary Data



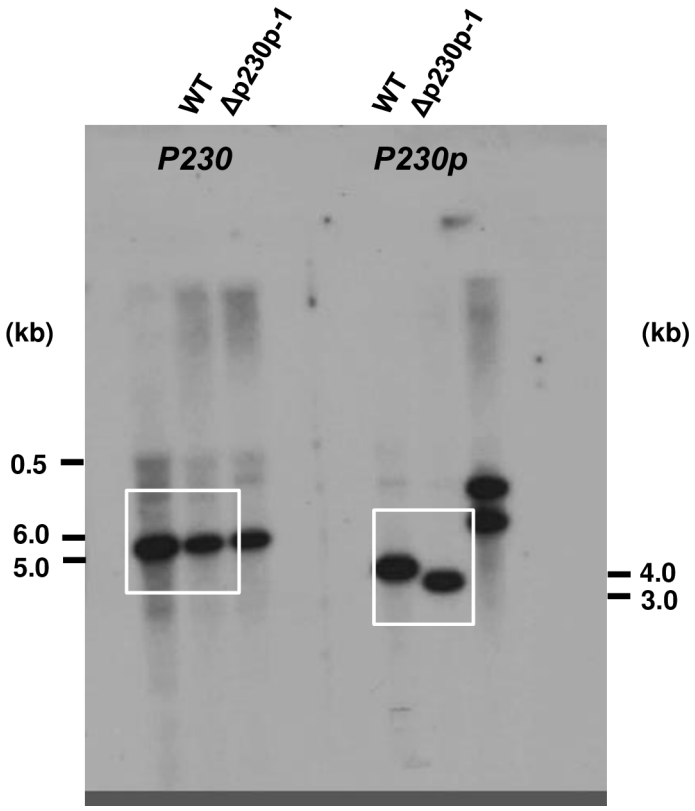
Supplementary figure 1. Analysis of expression of P230p , P230 and P48/45 in mature and activated gametocytes of P230p-GFP parasites and *PfΔp230p-2*. **A.** GFP-fluorescence in different stages of gametocyte development, stage II, III, IV and mature, stage V, gametocytes of a transgenic *P. falciparum* (3D7) line that expresses a C-terminal GFP-tagged version of *p230p* (*p230p* -gfp). Scale bar, 7µm**. B.** GFP-fluorescence in live male gametocytes of the P230p-GFP line 15-20min after activation. Scale bar, 7µm. **C**. Immunofluorescence analysis of fixed activated male gametocytes of the P230p-GFP line 15-20min after activation. Cells were fixed with methanol and labelled with rabbit anti-GFP antibody followed by goat anti-rabbit antibody secondary conjugated to Alexa Fluor® 488. Scale bar, 7µm. **D.** Immunofluorescence analyses of mature, stage V, *PfΔp230p-2* gametocytes ▸

after activation with fetal calf serum. Upper panel, female gametes 30 minutes after gametocyte activation. Unfixed parasites were labelled with mouse anti-P230 (MAb 63F2A2) or rat anti-P48/45 (MAb 85RF45.1) antibodies followed by secondary conjugated antibodies (i.e. anti-mouse or anti-rat IgG Alexa Fluor® 488 (green)). Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor® 488 (green) 0.7 s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7µm. Lower panel, Immunofluorescence analyses of male gametes 15 minutes after gametocyte activation in fetal calf serum. Cells were fixed with methanol and labelled with mouse anti-P230 (MAb 63F2A2) or rat anti-P48/45 (MAb 85RF45.1) antibodies followed by secondary conjugated antibodies (i.e. anti-mouse IgG Alexa Fluor® 488 (green) or anti-rat IgG Alexa Fluor® 594 (red)). Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor ® 488 (green) 0.7 s; anti-IgG Alexa Fluor ® 594 (red). 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7µm.

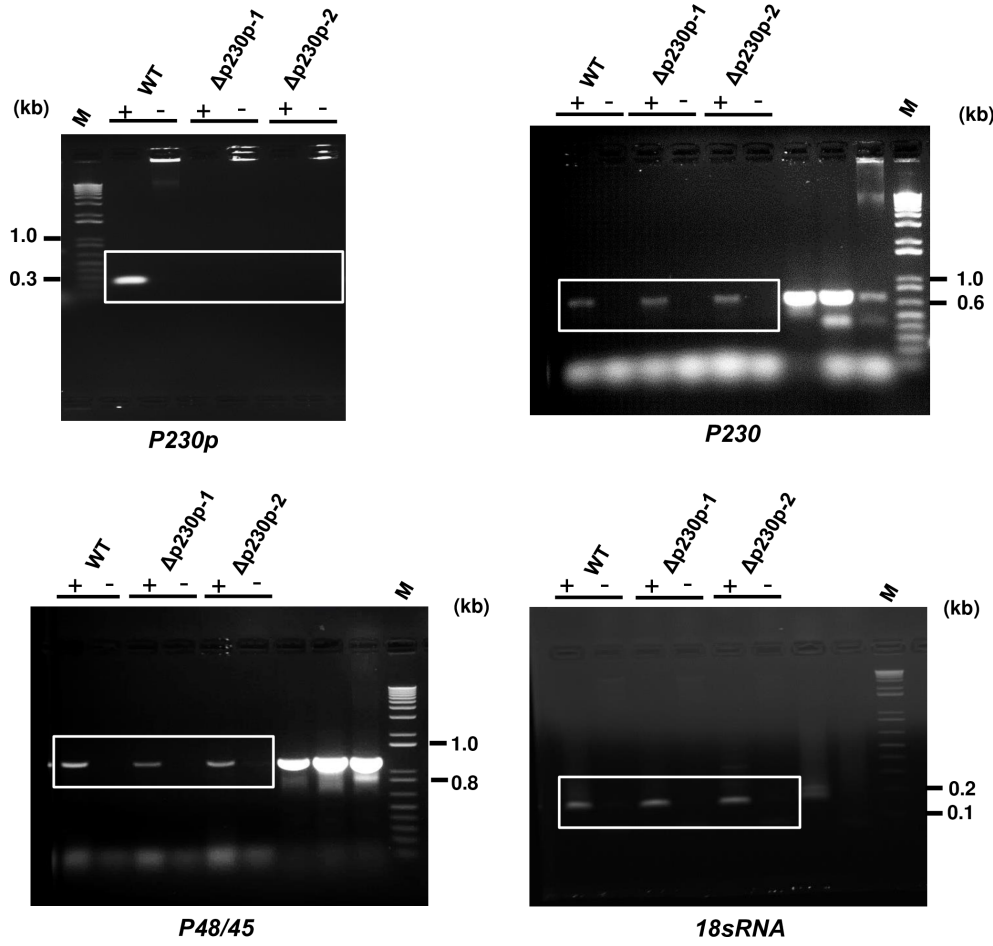


Supplementary figure 2. Generation and analysis of a *P. berghei* mutant line *(pbΔp230pΔp230)* lacking expression of P230 and P230p. **A.** Schematic representation of the generation of the *P. berghei* double gene-deletion mutant *pbΔp230pΔp230*. To generate this mutant the *p230* locus was disrupted in the existing *PbΔp230p* mutant (676m1cl1) which has a disrupted *p230p* locus containing a GFP-Luciferase expression cassette. To disrupt *p230* a DNA construct (pL1139) was used, which integrates into the P. berghei genome by double cross-over integration and replaces (part of) the *p230* locus with the selectable marker (SM) cassette containing *Toxoplasma gondii* dihydrofolate reductase/thimidylate synthase (*tgdhfr/ts*). Parasites of line 676cl1 were transfected with this construct (exp. 2764) using standard transfection technologies and selection with pyrimethamine. Selected parasites were cloned by limiting dilution and mutant 2764cl3 was used for genotype and phenotype analysis (see **B,C**). Location of primers (p) and PCR amplicons (in black) are indicated.

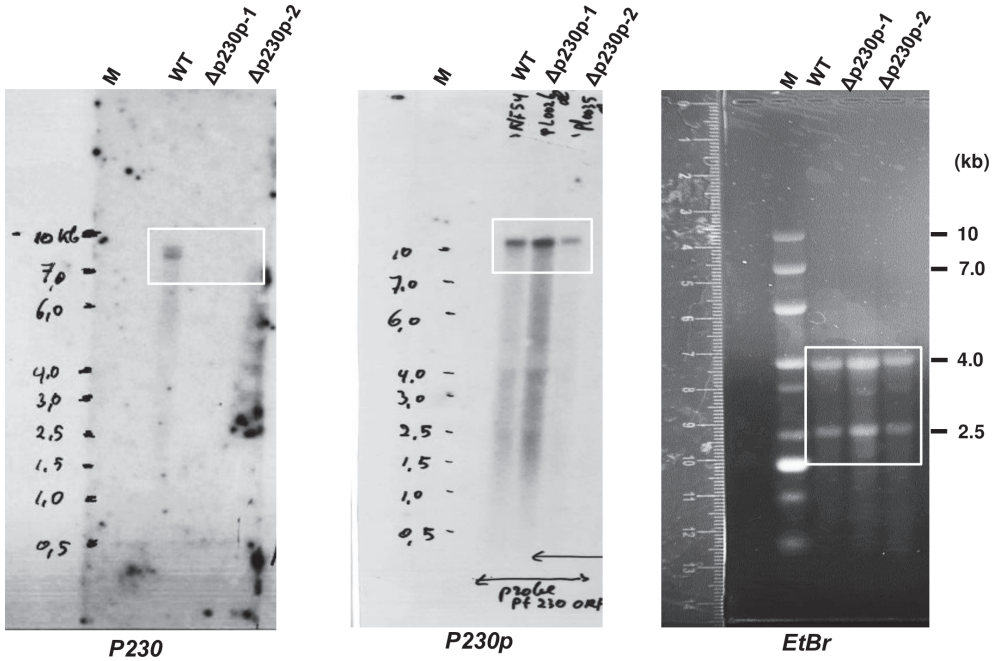
Primer sequences are shown **Table S1**. **B.** Diagnostic PCR (left, middle panel) and Southern analysis of PFG-separated chromosomes (right panel) confirms correct disruption of *p230* and *p230p* in line 2764cl3. 5'and 3'integration PCR (int), shows the expected bands of 881 bp and 1117 bp for disruption of *p230p* (primers p13/p14 and p17/p18) and 1224 bp and 1302 bp for *p230* (primers p21/p22 and P25/26). The GFP-Luciferase cassette in *p230p* was detected with primers p15/p16 (529bp) and the *tgdhfr/ts* SM cassette in the *p230* locus with primers p23/p24 (1039bp). Uncropped images of the gel images are shown in **Figure S6**. See **A** for primers, PCR amplicons and **Table S1** for primer sequences. Hybridization of PFG-separated chromosomes with the *tgdhfr* probe shows the integration of the *tgdhfr/ts* SM cassette in *230p* on chromosome 3. Hybridization with the probe against 3'UTR of the *P. berghei dhfr/ts* gene recognizes the endogenous *dhfr/ts* gene on chromosome 7 and the integrated casettes in *p230* and *p230p* in chromosome 3. Uncropped images of PFG-Southern analyses are shown in **Figure S**7. **C**. Exflagellation centres (EC, circles) as observed by light microscopy analysis of male gametocytes of *pbΔp230p* (line 676m1cl1), *pbΔp230* (line 310cl1) and pbΔp230pΔp230 (line 2764cl3) between 10 and 20 min after activation in live preparations in a Bürker cell chamber. Scale bar, 7µm. See also **Supplementary videos S7-S12** for the presence of exflagellation centres and **Table S4** for quantification of exflagellation centres in the mutant and wild type parasites.



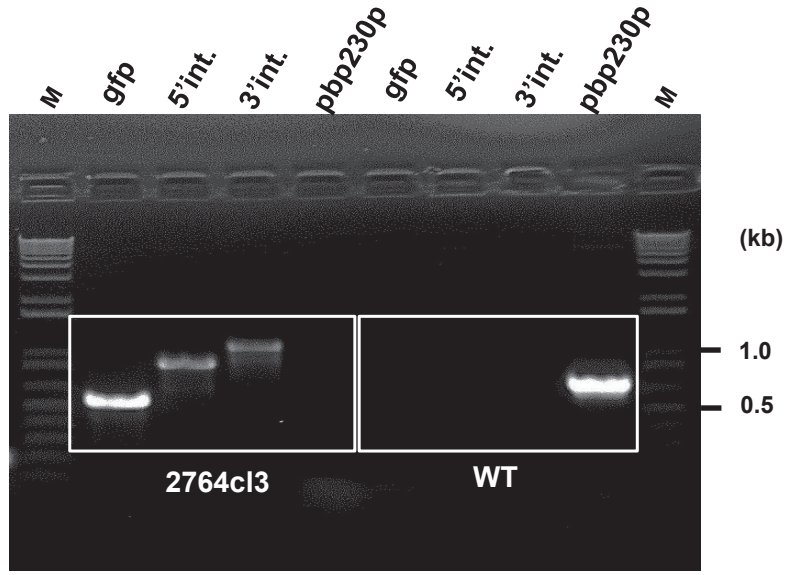
Supplementary figure 3. Unprocessed images of Southern blot analysis. The white boxes show the cropped image in **Figure 1B.** Molecular marker 1Kb plus ladder (M). DNA was electrophoresed in 0,8% agarose gel.

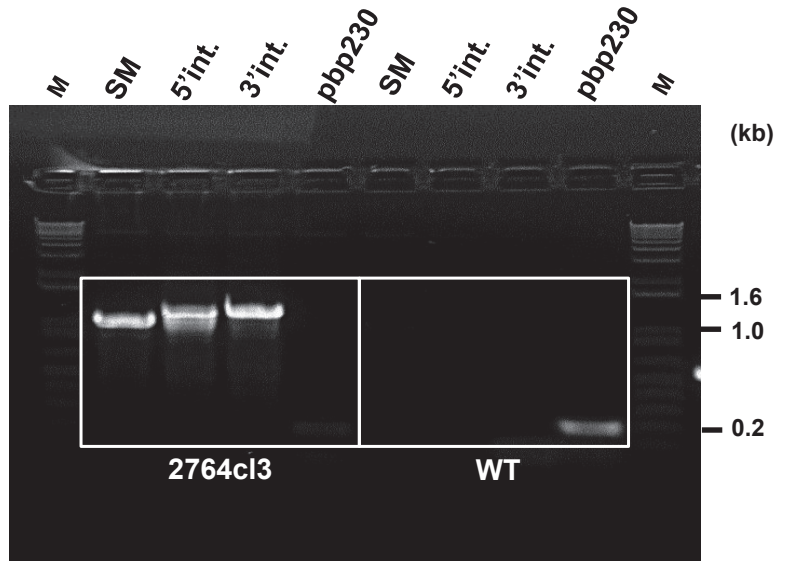


Supplementary figure 4. Unprocessed images of RT-PCR analysis. The white boxes show the cropped image in **Figure 1C** (left panel). Molecular marker 1Kb plus ladder (M). DNA was electrophoresed in 1% agarose gel.

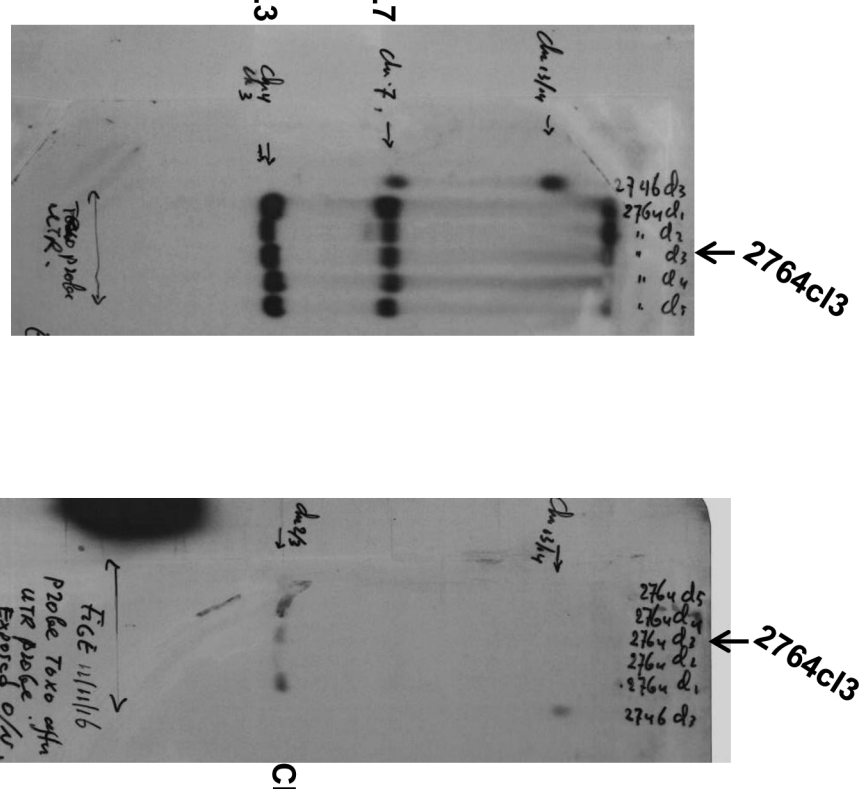


Supplementary figure 5. Unprocessed images of Northern blot analyses. The white boxes show the cropped image in **Figure 1C** (right panel). P230 and P230p panels show autoradiograph images after probing with 230 or 230p probes. EtBr panel shows electrophoresis gel stained with ethidium bromide *(EtBr)* of WT and *PfΔ230p* parasites. 5ug of RNA was seeded per lane. RNA Molecular weight marker (M) and RNA was electrophoresed in 1% agarose gel.





Supplementary figure 6. Unprocessed images of PCR analyses. The white boxes show the cropped image **Figure 2** (Left and Middle panel). Molecular marker; 1Kb plus ladder (M). DNA was electrophoresed in 1% agarose gel.



Supplementary figure 7. Unprocessed images of (PFG-separated) chromosomal Southern Blot analyses. The arrows indicated the lanes cropped out of this image and used in **Figure S2B** (Right panel). DNA was electrophoresed in 1% agarose gel.

Supplementary table 1. List of primers used in this study

Primer ID	Leiden code	Gene ID	Sequence	Product (bp)	Description
***pfΔp230p* genotyping**					
P1	8386	PF3D7_0209000	CGCTGAAGAATCTATTCCTC	745	Forward *pfs230*
P2	8387	PF3D7_0209000	CTATCCGAGGGGGTTAAATAC		Reverse *pfs230*
P3	7869	PF3D7_0208900	TTATTGGGCCCGTCGACGTTGATAAGGATAGTGTTTCAG	867	Forward HR2 *pf230p*
P4	7871	PF3D7_0208900	TCCTTAAGCTTTACGTAGGATTAATATTCCCATTAGG		Reverse HR2 *pf230p*
P5	8038	PF3D7_0208900	GATGATCTAAAAAAAGAAGAGTG	259	Forward *pf230p*
P6	8039	PF3D7_0208900	CATTACAATAACAAAATAAATGAAC		Reverse *pf230p*
P7	6068	PF3D7_1346700	ATTCCATATGAAAACAATGATTTTTGTAAGCCTAGC	1219	Forward *pfs48/45*
P8	6069	PF3D7_1346700	GGCGGCCGCTTACTATAGTAACTGTCATATAAGCAC		Reverse *pfs48/45*
P9	8733		GTTAAGGGAGTGAAGACGATCAGA	165	Forward *plasmodium 18sRNA*
P10	8734		AACCCAAAGACTTTGATTTCTCATAA		Reverse *plasmodium 18sRNA*
***pbΔp230pΔp230* Genotyping**					
P11	8658	PBANKA_0306000	AATGCACCATCGTATGTGATAG	715	Forward *pb230p*
P12	8659	PBANKA_0306000	CGTCCCATCTATGCTACTCAC		Reverse *pb230p*
P13	5510		GCAAAGTGAAGTTCAAATATGTG	881	Forward 5' Integration *pbΔ230p*
P14	7289		TAAAGCACAATATCTAGGATACTAC		Reverse 5' Integration *pbΔ230p*
P15	7295		ATAAGAATGCGGCCGC GATCTATGAGTAAAGGAGAAGAAC	529	Forward gfp::luc reporter cassette
P16	7294		CTTCCATCTTCAATGTTGTGTC		Reverse gfp::luc reporter cassette
P17	7922		GTCTCTTCAATGATTCATAAATAGTTGG	1117	Forward 3'Integration *pbΔ230p*
P18	5511		AGTGACTTTCAGTGAAATCGC		Reverse 3'Integration *pbΔ230p*
P19	8654	PBANKA_0306100	CAACATGTACTTAAGTTAGACTTAG	198	Forward *pbs230*
P20	8655	PBANKA_0306100	GGATTCATTAAAATATTTTCCATATTTATG		Reverse *pbs230*
P21	8656		GCCCAACAAAAGATTTAGGAAAT	1224	Forward 5' Integration *pbΔs230*
P22	6382		ATTTGTGTCTATATTACCAACTC		Reverse 5' Integration *pbΔs230*

Supplementary table 1. (continued)

Primer ID	Leiden code	Gene ID	Sequence	Product (bp)	Description
P23	4598		GGACAGATTGAACATCGTCG	1039	Forward tgdhfr/ts selectable marker
P24	4599		GTGTAGTCTGTGTGCATGTC		Reverse tgdhfr/ts selectable marker
P17	7922		GTCTCTTCAATGATTCATAAATAGTTGG	1302	Forward 3' integration *pbΔ230*
P26	8657		GCTTTCATATGTTGTATTAGTATTATCAC		Reverse 3' integration *pbΔ230*
P27	L692	PBANKA_0719300	CTTATATATTTATACCAATTG	561	Forward 3'UTR *pbdhfr*
P28	L693	PBANKA_0719300	GTTTTTTTTTAATTTTTCAAC		Reverse 3'UTR *pbdhfr*

Supplementary table 2. Published RNAseq and proteome data on expression of P230, P230p and P48/45 in male (m) and female (f) gametocytes

	Lasonder, E. et.al. 2016		Miao, J. et.al. 2017	Tao, D. et.al. 2014	Khan, SM. et.al 2005
Protein	**m/f ratio transcriptome[1]**	**m/f ratio proteome[2]**	**m/f ratio proteome[3]**	**m/f presence proteome[4]**	**m/f ratio proteome[5]**
P230p	54	54	male only	male only	male only
P230	4,4	0,5	1,1	in male and female	male only
P48/45	4,2	1,0	1,4	in male and female	4,1

[1] The ratio of RNAseq RPKM values in separated male and female gametocytes (Lasonder, E. et.al, 2016, Nucleic Acids Res 44(13):p.6087-101).
[2] The ratio of proteome spectra values in separated male and female gametocytes (Lasonder, E. et.al, 2016, Nucleic Acids Res 44(13):p.6087-101).
[3] The ratio of proteome spectra values in separated male and female gametocytes (Miao, J. et.al, 2017, Mol Cell Proteomics, 2017. **16**(4): p. 537-551.).
[4] The ratio of proteome spectra values in separated male and female gametocytes (Tao, D. et.al, 2014, Mol Cell Proteomics, 2014. **13**(10): p. 2705-24.).
[5] The ratio of proteome spectra values in separated male and female gametocytes (Khan, SM. et.al, 2005, Cell, 2005. **121**(5): p. 675-87).

Supplementary table 3. Oocyst and sporozoite production in *A. stephensi* mosquitoes of WT and *pfΔp230p* parasites

Lines	No. of oocyst mean (range)[1]	No. of spz (x10^3) mean (range)[2]
WT		
NF54 (7 exp.)	**18,73 (4-32)**	**56 (11-90)**
Δp230p-1		
0022cl1 (6 exp.)	0,39 (0,05-0,6)	0
0022cl5 (5 exp.)	0,38 (0,1-0,7)	0
Δp230p-2		
0035cl4 (6 exp.)	0,12 (0-0,5)	0

[1] Mean number of oocysts per mosquito at day 8 after feeding. Range corresponds to the mean number of oocyst in multiple experiments (5-7 exp. per line; 10-20 mosquitoes per exp.)
[2] Mean number of salivary gland sporozoites per mosquito at day 14 after feeding.
Range corresponds to the mean number of oocyst in multiple experiments (5-7 exp. per line; 10-20 mosquitoes per exp.)

Supplementary table 4. Exflagellation and fertilization rates of WT, *pbΔp230p*, *pbΔp230* and *pbΔp230pΔp230* parasites *in vitro*

Lines	% exflagellating males mean (SD)[1]	Exflagellation centers[2]	Fertilization rate (%) mean (SD)[3]
WT			
cl15cy1*	76-92 (85)	+++	59 (6,7)
pbΔp230p			
676m1cl1	50-92 (75)	+++	**55-80(10)**
pbΔp230			
310cl1*	**72-90 (80)**	+++	<0,1
323cl1*		+++	<0,1
pbΔp230pΔp230			
2764cl3 (n=2)	75-95 (85)	+++	<0,1 (0,01)

[1] Percentage of exflagellating male gametocytes between 10-20 minutes after activation.
[2] Presence of exflagellation centers between 10-20 minutes after activation. Exflagellation centers are counted in Bürker cel counter. +++: >90% of the exflagellating male gametocytes form exflagellation centers
[3] The fertilisation rate is the percentage of female gametes that develop within 24 hours into ookinetes in vitro.
* Data taken from van Dijk MR et.al, 2010, PLoS Pathog, 2010. **6**(4): p. e1000853

CHAPTER 5

A *P. falciparum* NF54 reporter line expressing mCherry-luciferase in gametocytes, sporozoites and liver stages

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Manuscript in preparation

Abstract

Transgenic malaria parasites expressing fluorescent and bioluminescent proteins are useful tools to interrogate malaria parasite biology and to quantify parasite-host interactions. Here we report the generation of a transgenic *Plasmodium falciparum* (*Pf*) NF54 line expressing a fusion gene of *mCherry* and *luciferase* under the control of the *etramp10.3* promoter. The *Pf* ETRAMP10.3 protein is related to the rodent *Plasmodium* UIS4 protein, which is also a member of the ETRAMP protein family. In rodent malaria *Plasmodium* species, the promoter of the *uis4* gene has been used to drive high transgene expression in liver-stages parasites. CRISPR/Cas9 methodology was used to insert the *mCherry-luc@ etramp10.3* expression cassette into the *Pf p47* gene locus. We demonstrate mCherry expression in gametocytes, sporozoites and liver-stages. While we did not detect mCherry above background levels in asexual blood-stage parasites, luciferase expression was detected in asexual blood-stages as well as gametocytes, sporozoites and liver-stages. Highest levels of reporter expression were detected in stage III-V gametocytes and in sporozoites. The expression of mCherry and luciferase in gametocytes and sporozoites makes this transgenic parasite line suitable to use in *in vitro* assays to examine the effect of inhibitors on gametocyte development and to analyse sporozoite biology.

Introduction

Transgenic rodent and human malaria parasites expressing fluorescent and bioluminescent proteins are used extensively to interrogate parasite biology and host-parasite interactions associated with malaria pathology and are used as tools to evaluate anti-parasite inhibitors and vaccines [1]. In comparison to transgenic rodent malaria parasites (RMP) only a relatively limited number of transgenic *Plasmodium falciparum* (*Pf*) parasites expressing fluorescent or luminescent proteins are available. Transgenic *Pf* parasites have been used to quantify blood-stage growth *in vitro* in standard growth inhibition assays [2], to quantify parasite development in the mosquito in standard membrane feeding assays to measure transmission-blocking (TB) activity and in high-throughput screening of TB compounds against *Pf* gametocytes. For the TB assays transgenic *Pf* (NF54 strain) parasite lines have been used that express a GFP-luciferase fusion protein under control of the strong constitutive *hsp70* [3] or the gametocyte-specific *pfs16* promoter [4]. In addition, a transgenic *Pf* NF54 has been created that express the GFP-luciferase fusion protein under control of the constitutive *eef1a* promoter [5]. This reporter line has been used in multiple studies to analyse liver infection in immune compromised and humanized mice, engrafted with human liver tissue [6-8].

In multiple RMP transgenic lines the promoter of the *uis4* gene has been used to drive expression of different transgenes, in sporozoites and liver-stages, such as genes encoding mCherry, ovalbumin or human malaria proteins [9-15] The *uis4* gene is highly transcribed in sporozoites and liver-stages and encodes a parasitophorous vacuole membrane (PVM) protein, that surrounds the parasite in the infected hepatocyte. Although *uis4* transcripts are translationally repressed in sporozoites [16, 17], the transcripts containing transgenes under control of *uis4* regulatory sequences are transcribed since translational repression in sporozoites is dependent on DNA sequences present within the *uis4* open reading frame [9, 16]. In this study, we generated a transgenic *Pf* parasite that expresses a fusion of the proteins mCherry and luciferase (mCherry-Luc) under the control of the promoter region of *etramp10.3* (PF3D7_1016900). We selected this promoter since *etramp10.3* is related to *uis4* which also belongs to the *Plasmodium etramp* gene family and both genes have the same syntenic genomic location. It has been previously reported that *etramp10.3* is expressed in *Pf* sporozoites as well as in blood- and liver-stages, where the protein is located at the PVM, similar to the PVM location of UIS4 in liver-stages of rodent malaria parasites [18].

We chose to generate an mCherry-expressing *Pf* line, as it could be used to visualise interactions of *Plasmodium* sporozoites with host-cells (e.g. immune cells or hepatocytes) which are often labelled with green fluorescent proteins. Moreover, we fused the mCherry gene to firefly luciferase as luciferase expression can be used to quantify parasite numbers (e.g. sporozoites and liver-stages) using simple and sensitive luminescence assays [19-21]. The mCherry-luciferase expression cassette was introduced, using a CRISPR/Cas9 methodology into the *p47* gene locus, a locus that has been previously used to introduce transgenes into the *Pf* genome [5, 22].

Results and discussion

Generation of a transgenic reporter line, mCherry-luc@etramp10.3, expressing a fusion protein of mCherry and Luciferase

Using CRISPR/Cas9 gene editing we created a transgenic *P. falciparum (Pf)* parasite line that contains an *mCherry-luciferase* fusion gene under control of 1.7 kb of 5'UTR of the *etramp10.3* gene (PF3D7_1016900). This expression cassette was introduced into the neutral *p47* gene locus (PF3D7_1346800). We used a previously described Cas9 construct (pLf0019), containing the Cas9 expression cassette and a *blasticidin* (BSD) drug-selectable marker cassette [23] in combination with a sgRNA donor-DNA plasmid (pLf0049)(**Figure S1**). This plasmid contains the *p47* targeting sequences, the *mCherry-luc@ etramp10.3* expression cassette and a h*dhfr-yfcu* drug-selectable marker cassette (See **Figure 1** and Materials and Methods section for details of the generation of the constructs). Donor DNA plasmid pLf0049 aims to integrate the reporter *mCherry-luc@etramp10.3* cassette into the *p47* gene locus by double cross-over homologous recombination (**Figure 1**).

Transfection of *Pf* NF54 parasites was performed using synchronized ring-stage parasites that were transfected with ~50 µg of each circular plasmid (Cas9 and sgRNA/donor-DNA constructs; see Materials and Methods section) and selection of transformed parasites containing both plasmids (Cas9 and sgRNA/donor-DNA constructs) was performed by applying 'double' positive selection using the drugs WR99210 and BSD until parasites were detectable by thin blood-smear analysis (between day 14 to 26 post transfection). Subsequently, parasites were cultured for 2-4 days without drugs, followed by the application of negative (5-FC) selection to eliminate parasites that retain transfection constructs (i.e. donor-DNA) as episomal plasmids and to enrich for parasites in which the donor-DNA construct has integrated into the parasite genome. Subsequently, drug-selected parasites were cloned by limiting dilution. Genotyping of two clones by Southern analysis and PCR revealed the correct integration of the *mCherry-luc@etramp10.3* cassette into the *Pf* genome (**Figure 1B**). Blood-stages of both clones had growth rates comparable to blood-stages of the parent wild type (WT) NF54 strain (**Figure 1C**). For further phenotype analysis (as described below) we selected clone 3, which we confirmed as not retaining episomal plasmid by Southern blot analysis (**Figure 1B**).

mCherry and luciferase expression in mCherry-luc@etramp10.3 blood-stages

We analysed mCherry expression in cultured asexual blood-stages and gametocytes of the *mCherry-luc@etramp10.3* line. In all asexual blood-stages (ring-forms, trophozoites and schizonts) we detected mCherry signals that were indistinguishable from the background fluorescence of uninfected red blood cells (**Figure 1D**). We next examined gametocytes cultures of the *mCherry-luc@etramp10.3* line, which produced comparable numbers of mature stage V male and female gametocytes as WT NF54 parasites (**Table S1**). In *mCherry-luc@etramp10.3* stage III-V gametocytes a clear mCherry signal was detected (**Figure 1E**). Weak mCherry signals were detectable in at least 20% of stage II gametocytes, increasing to more than 95% of stage III-V gametocytes, which were strongly mCherry positive.

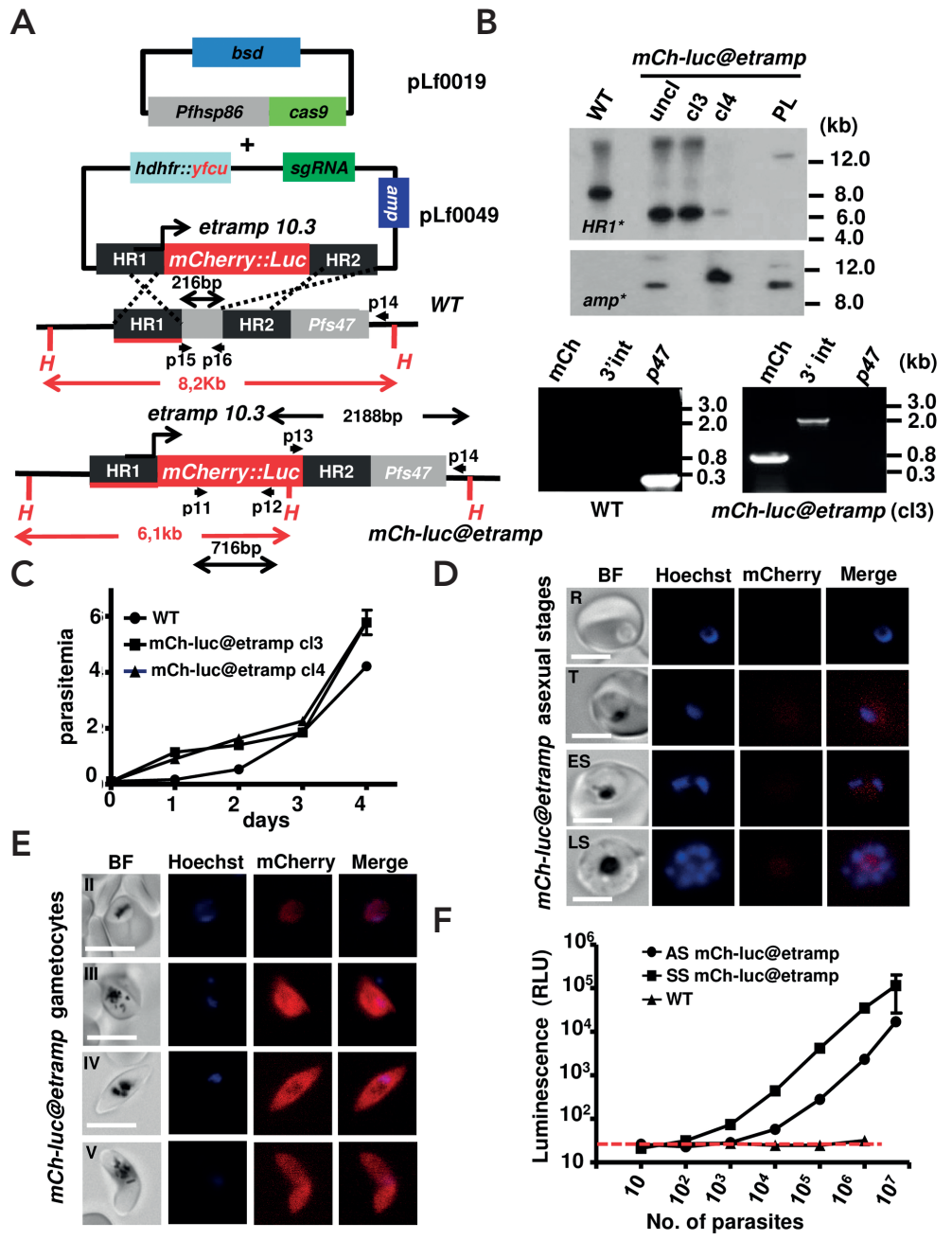

Figure 1. A *P. falciparum* reporter line expressing mCherry-luciferase under control of the *etramp10.3* promoter: generation, genotyping and analysis of expression of mCherry and luciferase in asexual blood stages and gametocytes. **A.** Schematic representation of the Cas9 (pLf0019) and sgRNA/donor (pL0049) constructs generated to introduce the mCherry-luciferase expression cassette into the *P. falciparum* (*Pf*) *p47* gene locus. The mCherry-luciferase fusion gene is under the control of the promoter of the *etramp10.3* gene. *p47* homology regions (HR1, HR2) used to introduce the donor DNA (i.e. *gfp* expression cassettes), location of primers (p) and sizes of restriction fragments (H: *Hpa*I; in red) ▸

and PCR amplicons (in black) are indicated. Primer sequences (shown in black and bold) are shown in Table S2. *bsd* – blasticydin selectable marker (SM); *hdhfr::yfcu* – SM in donor plasmid. *mCh-luc@etramp* – the final reporter line *mCherry-luc@etramp10.3*. **B.** Southern analysis of *Hpa*I restricted DNA (upper panel) and diagnostic PCR (lower panel) to confirm correct integration of construct pLf0049 into the *p47* locus. Digested DNA of wild type (WT), transfected, uncloned (uncl) parasites, selected parasite clones 3 and 4 and plasmid (PL) of *mCh-luc@etramp* was hybridized with a probe targeting the homology region 1 of *p47* (HR1; primers p3/p4; see **A**), identified the expected different-sized DNA fragments in wild type (WT) and *mcherry-luc@etramp10.3* (8.2 kb and 6.1 kp shown in red in **A**). The absence of hybridisation of digested DNA hybridized with a probe for *ampicillin* (*amp*) gene confirms absence of donor-DNA plasmid and single cross-over events in clone 3.. Diagnostic PCR of *mCh-luc@etramp* clone 3 confirms the presence of the *mCherry-luciferase* gene (lane 1; primers p11/p12; expected size: 716bp), correct 3' integration (lane 2; primers p13/p14; expected size: 2188bp) and absence of the *p47* gene (lane 3; primers p15/p16); expected size: 216bp). Primer locations and product sizes are shown in **A** and primer sequences in **Table S1**). **C.** Growth of asexual blood-stages of the *mcherry-luc@etramp10.3* lines (clone 3 and 4) and WT parasites in semi-automated culture system for a period of 4 days. Cultures were initiated with a parasitemia of 0.5%. **D.** Fluorescence microscopy analysis of live *mcherry-luc@etramp10.3* asexual blood-stages. No mCherry fluorescence signal above background were detected in the different stages. R: rings; T: trophozoites; ES: early schizonts; LS: late schizonts;. Nuclei were stained with Hoechst-33342. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (mCherry 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain)). Scale bar, 4µm. **E.** Fluorescence microscopy analysis of mCherry expression in live *mcherry-luc@etramp10.3* gametocytes. Gametocyte stage II, III, IV and V are shown. Nuclei were stained with Hoechst-33342. All pictures were recorded with standardized exposure/gain times to visualize differences in fluorescence intensity (mCherry 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain)). Scale bar, 7µm. **F.** Correlation between luminescence levels and number of parasites in serial dilutions series of asexual blood stages (AS) and gametocytes stage III/IV (SS) of the *mcherry-luc@etramp10.3* line. Wild type NF45 parasites (WT) were used as a control. Red dotted line: luminescence value of uninfected cells. The mean luminescence value of triplicate samples is shown; error bars represent the standard deviation. Correlation coefficient r (two-tailed Spearman's test: 10^3-10^7 parasites): 1.00; p=0.016* for AS and 1.0; p=0.016 * for SS.

We also examined expression of the *mCherry-luciferase* in blood-stage parasites by performing luminescence assays. Unlike mCherry, luminescence signals obtained from mixed asexual blood-stage parasites were significantly higher than uninfected cells ($p < 0.0005$ in culture wells with more than 10^4 parasites) (**Figure 1F**). Gametocytes (stage IV/V) had on average 30-fold higher luminescence values (3 exp.; range 15-60 fold) compared to mixed asexual stages. The luminescence values obtained from *mCherry-luc@etramp10.3* gametocyte (IV/V) or mixed asexual stage dilution series demonstrate a linear relationship between the number of parasites and signal intensity in the range of $1x10^3$ to $1x10^7$ parasites for gametocytes and $1x10^4$ to $1x10^7$ for asexual blood-stage parasites (**Figure 1F**). The high activity of the *etramp10.3* promoter in gametocytes is in agreement with the high levels of *etramp10.3* transcripts and ETRAMP10.3 protein previously reported in gametocytes by genome-wide analyses of gene expression. Peak in *etramp10.3* transcript abundance was observed in stage III gametocytes [24] and ETRAMP10.3 is detected in proteomic analyses of (male and female) gametocytes [25, 26] and is more abundant in gametocytes compared to asexual blood-stages. The expression of ETRAMP10.3 in asexual blood-stages has been reported after proteomic analyses [27, 28] and has been

confirmed by immunofluorescence analysis using anti-ETRAMP10.3 antibodies [18]. Moreover, unsuccessful attempts to delete the gene *etramp10.3* indicates that it is essential during asexual blood-stage development [18]. This vital role of ETRAMP10.3 during blood-stage development is in contrast to rodent *Plasmodium* UIS4, which is dispensable for blood-stage development [29, 30]. In previous studies using rodent parasites, where the *usi4* gene has been replaced with the *etramp10.3* gene, it was demonstrated that *Pf* ETRAMP10.3 is unable to complement the essential function of UIS4 in *P. yoelii* liver-stages [18]. These observations indicate that UIS4 and ETRAMP10.4 may have different or only partially overlapping roles in rodent and human malaria parasites.

mCherry and luciferase expression in mCherry-luc@etramp10.3 sporozoites

We examined mCherry expression in oocysts and sporozoites collected from *Anopheles stephensi* mosquitoes fed with *mCherry-luc@etramp10.3* gametocytes (using the standard membrane feeding assay). *mCherry-luc@etramp10.3* parasites produced oocysts and sporozoites in numbers that were in the same range as the parent wild type (WT) NF54 parasites (**Table S1**). No mCherry signals, distinguishable from background, were detected in maturing oocysts containing sporozoites (at day 8 and 11 after feeding) (**Figure 2A**). Salivary gland sporozoites, however, were clearly mCherry positive (**Figure 2B**, **Figure S2**). The activity of the *etramp10.3* promoter in sporozoites is in agreement with detection of ETRAMP10.3 protein in proteomes of sporozoites [31, 32]. Expression of mCherry-luciferase in sporozoites was also confirmed by luminescence assays (**Figures 2B,E** and **Figure 2B**) and luminescence signals from a dilution series of purified sporozoites exhibit a linear relationship between sporozoite number and luminescence intensity in the range of $1.25x10^4$ to $5x10^4$ sporozoites. We also compared the luminescence signals of *mCherry-luc@etramp10.3* sporozoites ($5x10^4$) with that of transgenic sporozoites expressing a GFP-luciferase fusion protein under control of the *eef1α* promoter [5]. The luminescence signal of *mCherry-luc@etramp10.3* sporozoites was 14-fold higher than the luminescence signal obtained from the same number of *GFP-luc@eef1α* sporozoites (**Figure 2E**).

mCherry and luciferase expression in mCherry-luc@etramp10.3 liver-stages

Cultured cryopreserved primary human hepatocytes were infected with $5x10^4$ *mCherry-luc@etramp10.3* or *GFP-luc@eef1α* sporozoites per well of a 96-wells plate. Development of liver-stages of these two transgenic lines at day 2, 4 and 6 after infection was compared with WT liver-stages by immunofluorescence using a rabbit polyclonal antibody against the cytoplasmic protein PfHSP70. The percentage of infected hepatocytes, the size of the liver-stages and the intensity of HSP70 staining were comparable between the transgenic and WT parasites at day 4 after infection and liver-stages of both transgenic lines were comparable at day 6.

Expression of mCherry in *mCherry-luc@etramp10.3* liver-stages was analysed at day 3, 4 and 5 after hepatocyte infection by immunofluorescence assay using anti-mCherry

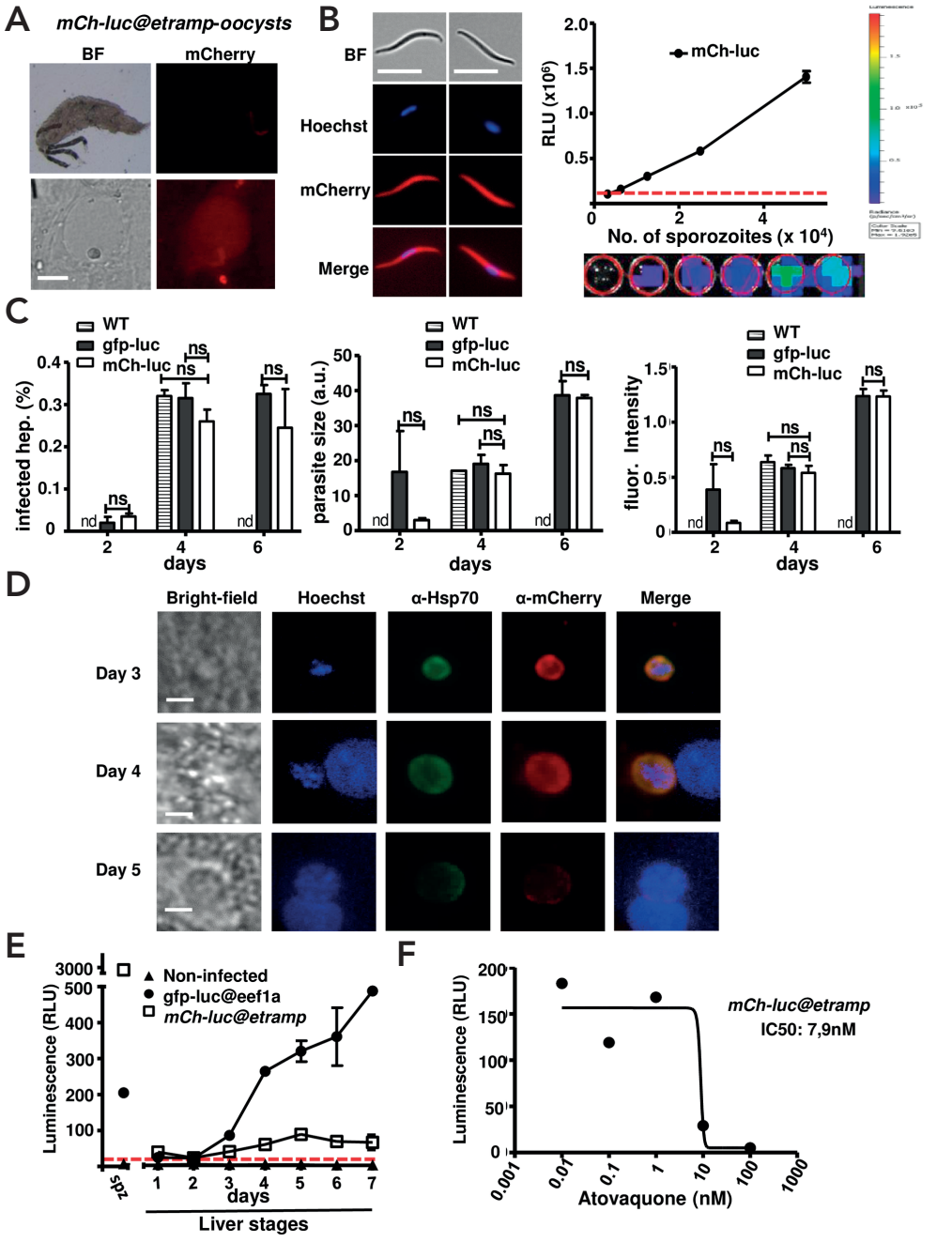

Figure 2. A *P. falciparum* reporter line expressing mCherry-luciferase under control of the *etramp10.3* promoter: analysis of mCherry and luciferase expression in oocyst, sporozoites and liver stages. **A.** Fluorescence microscopy analysis of mCherry expression in live *mcherry-luc@etramp10.3* oocyst in *A. stephensi* mosquitoes at day 10 after infection. No mCherry fluorescence signal above background was detected. Upper panel: complete midgut with 20to 30 oocysts. Lower panel: a single oocyst. BF - bright field. Scale bar, 20µm. **B.** *Left panel*: Fluorescence microscopy analysis of mCherry expression in live *mcherry-luc@etramp10.3* in salivary glands sporozoites collected at day 21 after infection of mosquitoes. Nuclei were stained with Hoechst33342. BF - bright field. Scale bar, 20µm. *Right panel*: Luminescence signals and correlation between luminescence levels and number of parasites in serial dilutions series of salivary gland sporozoites. Red dotted line: luminescence value of samples without sporozoites. The mean luminescence value of duplicate samples is shown; error bars represent the standard deviation. Correlation coefficient r (two-tailed Spearman's test) : 0.99; p=0.016*. **C.** Development of liver-stages of *mCherry-luc@etramp10.3* and *GFP-luc@eef1α* parasites in cultured cryopreserved primary human hepatocytes which were infected with $5x10^4$ sporozoites. Liver-stage development was analysed at day 2, 4 and 6 after infection and compared with WT liver-stages by immunofluorescence using antibodies against the cytoplasmic protein *Pf*HSP70. The percentage of infected hepatocytes (left graph), the size of liver-stages (middle graph; mean surface area; arbitrary units - a.u.) and the fluorescence intensity of *Pf*HSP70 staining (right graph; arbitrary units a.u. $x10^6$) were comparable between the transgenic and WT parasites at day 4 after infection and liver-stages of both transgenic lines were comparable at day 6. At least 20 parasites were assessed at each time point. nd: non determined. Significance values (unpaired two-tailed *t* test): n.s. – not significant **D.** Immunofluorescence analysis of mCherry expression in fixed liver-stages in cryopreserved human liver hepatocytes. Hepatocytes were fixed at days 3, 4 and 5 after sporozoites infection and were stained with rabbit anti-*Pf*HSP70 and goat anti mCherry antibodies. Secondary conjugated antibodies used: anti-IgG from rabbit Alexa Fluor® 488 (green) or anti-IgG from goat Alexa Fluor ® 594 (red). Nuclei stained with Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor® 488 (green) 0.7 s; anti-IgG Alexa Fluor ® 594 (red) 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 10µm. **E.** Luminescence levels in sporozoites (spz) and in liver-stages from *mCherry-luc@etramp10.3* and *gfp-luc@eef1a* lines. Cultured cryopreserved primary human hepatocytes were infected with $5x10^4$ sporozoites and luminescence was measured during a 7-day period. Uninfected hepatocytes were used as a control. Red dotted line: luminescence value of uninfected cells. The mean luminescence value of duplicate samples is shown; error bars represent the standard deviation. **F.** Sensitivity of *mCherry-luc@etramp10.3* liver-stages to atovaquone (ATQ). Inhibition of liver stage development was determined by measurement of luminescence at day 4 after infection of cultured cryopreserved primary human hepatocytes with $5x10^4$ sporozoites of development. The IC50 value (7.9nM) was calculated by non-linear regression using GraphPad Prism software package 5.04.

antibodies and mCherry signals were detected at all days (**Figure 2D**). Expression of mCherry-luciferase in liver-stages was also confirmed by luminescence assays (**Figure 2E**). We compared the luminescence signals of *mCherry-luc@etramp10.3* liver-stages with those of *GFP-luc@eef1α* at the same point of development. The luminesce signals of *mCherry-luc@etramp10.3* liver-stages, while significantly higher than background levels with a peak of expression at day 5, were 2.2 to7.6 fold lower at the same time point of development between day 3 and 7 after infection of the hepatocytes (**Figure 2E**).

To determine if *mCherry-luc@etramp10.3* parasites could be used in a plate-based assay to test drug sensitivity of liver-stage parasites, we performed an drug assay using atovaquone, which has potent activity against developing liver stage parasites [33]. The inhibition of *in vitro P. falciparum* liver-stage development was determined by measurement of luminescence of *mCherry-luc@etramp10.3*-infected hepatocytes maintained in 96-well plates and incubated with a serial dilution of atovaquone. Atovaquone was added to the 96-well cultures 3 hours after adding $5x10^4$ sporozoites to cultures of primary human hepatocytes and the cultures were allowed to proceed for

4 days after which luminescence was determined. In this assay we determined an IC50 value of atovaquone to be 7.9 nM (**Figure 2F**), which is close agreement to the previously established IC50 value of atovaquone against liver-stages [34].

The development and expansion of reporter lines in *P. falciparum* would increase the range of analyses that could be performed, both to interrogate *P. falciparum* gene function at different points of development and to permit the miniaturization and rapid screening of compounds or immune sera that target the parasite at different points of development. Indeed, mCherry-luciferase expressing sporozoites such as those described in this study could be used to better understand the interactions of sporozoites and host cells (i.e. in the skin) as well as to examine the action of drugs or vaccines that target gametocyte or liver stage parasites.

5

Materials and methods

In vitro cultivation of P. falciparum blood stages

P. falciparum (*Pf*) parasites from the NF54 strain were used [23]. Parasites were cultured using standard culture conditions in a semi-automated culture system as described[23] Fresh human serum and human red blood cells (RBC) were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria research and microbiology tested for safety). RBC of different donors were pooled every two weeks, washed twice in serum free RPMI-1640 and suspended in complete culture medium to 50% haematocrit. Human serum of different donors were pooled every 4-6 months and stored at -20°C until required. *Pf* gametocytes cultures were generated using standard culture conditions with some modifications as described[23]. Briefly, parasites from asexual stage cultures were diluted to a final parasitemia of 0.5% and cultures were followed during 14 days without refreshing RBC. At day 14 the cultures were analysed for mature, stage V, gametocytes.

In addition, a transgenic *P. falciparum* (NF54) was used that contains a reporter cassette with a fusion gene of GFP and luciferase (GFP-Luc) under control of *eukaryotic elongation factor 1 alpha* (*eef1a*) promoter [5].

Generation of the mcherry-luc@etramp10.3 parasite line

To create the reporter line we used a previously described Cas9 construct (pLf0019), containing the Cas9 expression cassette with a *blasticidin* (BSD) drug-selectable marker cassette [23] in combination with a sgRNA donor-DNA plasmid (pLf0049). The sgRNA-donor DNA construct (pLf0049) contains a h*dhfr-yfcu* drug-selectable marker (SM) cassette for selection with the drug WR99210. To generate pLf0049, the intermediate plasmid pLf0039 (**Figure S1**) was modified by introducing two homology regions targeting of *p47* (PF3D7_1346800). Homology region 1 (HR1) was amplified using primers P1/P2 and homology region 2 (HR2) with P3/P4 from *Pf* NF54 genomic DNA (see **Table S2** for primer details). HR1 was cloned in pLf0039 using restriction sites *Stu*II/*Sac*II and HR2 using

*Apa*I/*Hind*III. Subsequently, a guide RNA sgRNA targeting the *p47* locus (gRNA019) was selected using the Protospacer software (alphaversion;https://sourceforge.net/projects/protospacerwb/files/Release/), based on the best off targets hits score throughout the genome given by Protospacer and the total number of mismatches of the sgRNA with respect to the PAM site. A 20 bp guide sgRNA sequence (using the primers P5/P6), flanked on both sides by a 15 bp DNA sequence necessary for In Fusion cloning (HD Cloning Kit; Clontech), was annealed and used to replace the BtgZI adaptor in pLf0039 as previously described [35], resulting in pLf0047. The plasmid pLf0047 was digested with *Bln*I and *Nru*I to confirm successful cloning of the sgRNA and the correct sequence of the sgRNA (using primes P7/P8) confirmed by Sanger sequencing. An additional intermediate plasmid, pLf0130 (**Figure S1**) was modified by replacing the existing promotor region of the *gapdh* gene (PF3D7_1462800) by the promoter region of *etramp10.3* promoter (PF3D7_1016900) of a reporter cassette containing mCherry fused to luciferase with the 3'UTR region of histidine-rich protein II (GeneID PF3D7_0831800; 626 bp obtained by digestion of an intermediate plasmid pLf0053 ([23] with restriction sites *Apa*I/*Xba*I). The *etramp10.3* promoter region (1.7kb) was amplified from *Pf* NF54 genomic DNA using the primers P9/P10 and cloned into plf0130 using restriction sites *Sac*II/*Xho*I or *Kpn*I. Next, to obtain the complete *mcherry-luc @etramp10.3* expression cassette, pLf0130 was digested with *Apa*I/*Sac*II and this cassette was cloned in pLf0047 using the same enzymes, resulting in the final construct pLf0049.

5

Isolation of plasmids for transfection and transfection of synchronized ring stage parasites was performed as described [23] and parasites were transfected with a mixture of ~50 µg of each circular plasmid (Cas9 and sgRNA/Donor DNA construct). After transfection, parasite cultures were maintained under standard culture conditions in the semi-automated culture system (see above). Selection of transformed parasites was performed by applying 'double' positive selection 24 h after transfection using the drugs WR99210 (2.6 nM) and BSD (5 µg/ml) as described [23]. Drug pressure in the cultures was maintained until thin blood-smears were parasite-positive (usually after 14 to 26 days). Positive selection will select for the parasites that were transfected successfully with both plasmids (Cas9 and sgRNA/Donor constructs). Subsequently, both drugs were removed from the cultures for 2-4 days, followed by applying negative selection by addition of 5-Fluorocytosine as described [23] in order to eliminate parasites that retained the crRNA/Donor construct as episomal plasmid and enriching for the transfected parasites containing the donor DNA integrated into the genome. Negative drug pressure in the cultures was maintained until thin blood-smears were parasite-positive (usually after 7 days). After negative selection infected RBC (iRBC) were harvested from cultures with a parasitemia of 4 to 10% for genotyping by diagnostic PCR and Southern analysis (see next sections). Subsequently, selected parasites were cloned by limiting dilution as has been described previously [23]. Cloned parasites were transferred in 10 ml culture flasks at 5% haematocrit and cultured under standard culture conditions (see above) in the semi-automated culture system for collection of parasites for further genotype and phenotype analyses (see next section).

Genotyping of mcherry-luc@etramp 10.3 parasites

For genotyping diagnostic PCR and Southern analysis of digested DNA were performed using DNA isolated from iRBC obtained from 10ml cultures (parasitemia 3 - 10%). DNA was isolated as described [23]. Correct integration of the donors constructs was analysed by PCR amplification of the mCherry-luciferase gene (primers P11/P12), the fragment for 3' integration (3'int; primers P13/P14) and the *p47* gene (ORF; primers P15/P16). The PCR fragments were amplified using Go-taq® DNA polymerase (Promega) following standard conditions with an annealing temperature of 56°C for 20 sec and a elongation step of 72°C for 4 min. All other PCR settings were according to manufacturer's instructions.

Southern blot analysis of digested DNA was performed with *Hpa*I digested genomic DNA (4 hrs at 37°C). Digested DNA was hybridized with probes targeting the *p47* homology region 1 (HR1), amplified from NF54 genomic DNA by PCR using the primers P1/P2 and a second probe targeting ampicillin (Amp) gene, obtained by digestion of the intermediate plasmid pLf0040 with *Aat*II/*Pvu*I (550bp).

Phenotype analysis of of mcherry-luc@etramp 10.3 parasites

Asexual blood stages: The growth rate of asexual blood stages (parasitemia) was monitored by FACS analysis of iRBC stained with the DNA-specific dye Hoechst-33258 in 1 ml of PBS by adding 4 µl of a 500 µM stock-solution (final concentration 2 µM), as has been described previously[23]. mCherry expression of asexual blood stages was analysed by standard fluorescence microscopy. In brief, 200 µl samples of iRBC were collected from 10 ml cultures with a parasitemia between 4 and 10% and stained with the DNA-specific dye Hoechst-33342 by adding 4 µl of a 500 µM stock-solution (final concentration 10 µM) for 20 min at 37°C. Subsequently, a 5 µl was placed on a microscopic slide (mounted under a cover slip) and fluorescence in live iRBC analysed using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software and with the following exposure times: mCherry 0.6 sec; Hoechst-33342 0.136 s; bright field 0.62 s (1x gain).

Luciferase expression was determined in asexual blood stages. A serial dilution of asexual blood stages was prepared from parasites samples that were collected (in triplicate) from asexual blood stage cultures with a final number of $5x10^6$ parasites per sample. These were diluted with RPMI-1640 culture medium containing uninfected RBC (5% haematocrit) to prepare (triplicate) samples with $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$ parasites, respectively. Samples of 40 µl containing only uninfected RBC (5% haematocrit) were used as controls. The cells were pelleted by centrifugation (800g; 30s) and were lysed with 40 µl of cell culture Lysis 5X reagent from Promega (1 in 5 dilution in miliQ water). The complete lysates were collected in black 96-well plate (flat bottom) and luciferase activity was measured after adding 50 µl of the Luciferase substrate (Luciferase Assay System Promega). Luciferase activity (in relative light units; RLU) was measured using the Glomax multi detection system Luminometer (Promega) and the Instinct software (Promega).

Gametocytes: Gametocyte production (stage V male/female gametocytes) and exflagellation of male gametocytes were analysed in gametocyte cultures established as described previously [36]. For analysis of mCherry expression, stage II -V gametocytes were collected at days 7, 9, 11 and 14. Samples (200 µl) were collected, pelleted by centrifugation (800 *g*; 30 s) and stained with Hoechst-33342 for mCherry expression analysis using fluorescence microscopy as described for asexual blood stages. Luciferase expression was determined in gametocytes (stage III-IV) collected at day 11. A similar serial dilution of gametocytes was prepared as was done as described for asexual blood stages and luciferase activity in gametocytes was determined as described for the asexual blood stages.

Oocysts and sporozoites: For analysis of mosquito stages (oocysts and sporozoites), *Anopheles stephensi* mosquitoes were infected with day 14 gametocytes cultures using the standard membrane feeding assay (SMFA) [37, 38]. Oocysts numbers and mCherry expression in oocysts was determined at day 8 and 10 after infection. mCherry expression was analysed using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software and with the following exposure times: mCherry 0.6 sec; Hoechst-33342 0.136 s; bright field 0.62 s (1x gain). Collection of salivary gland sporozoite for counting numbers and expression of mCherry was performed at day 14 and 21 after feeding. For counting sporozoites, salivary glands from 30-60 mosquitoes were dissected, collected in 100 µl of RPMI-1640 pH 7.2 and homogenized using a grinder. Sporozoites were counted using a Bürker cell counter using phase-contrast microscopy. For mCherry expression, the isolated sporozoite were pelleted by centrifugation (800 *g*; 5 min). The pellet was suspended in 40 µl 1X PBS and sporozoites stained with Hoechst-33342 (10 µM) for 30 min at 37°C. Of this solution, 5 µl was placed on a microscopic slide (mounted under a cover slip) and fluorescence of sporozoites in live was analysed using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software and with the following exposure times: mCherry 0.6 sec; Hoechst-33342 0.136 s; bright field 0.62 s (1x gain).

Luciferase activity in sporozoites was determined in duplicate samples (total volume of 40 µl of RPMI) of a serial dilution of $0.31x10^4$ – $5.0x10^4$ salivary glands sporozoites. 50µl of D-Luciferin (0,4mg/ml; Perkin Elmer Life Sciences, Waltham, USA) was added to the 40µl of diluted sporozoites in a black 96-well plate (flat bottom). The *in vivo* imaging system Lumina (Caliper Life Sciences, USA) was used to measure luciferase activity. Imaging data were analysed using the Living Image® 4.5.5 software (Caliper Life Sciences, USA). Bioluminescence images were acquired with a 12,5 cm field of view (FOV), medium binning factor and an 'auto-exposure' time of maximum 2 minutes.

Liver stages: Liver stages were cultured *in vitro* using cryopreserved primary human hepatocytes obtained from Tebu-bio (Tebu-bio.com – Life science Research) and thawed according to the instructions of Sekisui/Xenotech (Sekisui XenoTech, LLC; Kansas City). Cells were cultured in Williams's E culture medium supplemented with 10% FCS, 1% penicillin-

streptomycin, 1% fungizone, 0,1IU/ml insulin and 70μM hydrocortisone 21-hemisuccinate (Sigma). Hepatocytes were seeded in Greiner clear bottom white 96-well plates at a density of 5x 10^4 cells per well, two days before infection with sporozoites as described previously [39]. Sporozoites were collected from infected mosquitoes at day 21 after as described above and hepatocyte cultures (at 37°C) were infected with 5x10^4 sporozoites per well. Three hours (hrs) after the addition of sporozoites, the cultures were washed three times with 1X PBS to remove mosquito material as well as sporozoites and complete Williams's E medium was added and cultures which were incubated overnight at 37°C. The day after, the culture medium was replaced and then was changed every 48 hrs until day 7.

Cultured cryopreserved primary human hepatocytes were infected with 5x10^4 *mCherry-luc@etramp10.3* and *GFP-luc@eef1*α sporozoites per well of a 96-wells plate. Development of liver-stages of these two transgenic lines at day 2, 4 and 6 after infection was compared with WT liver-stages by immunofluorescence using antibodies against the cytoplasmic protein *Pf*HSP70 (rabbit, anti-*Pf*HSP70; 1:75 dilution of 1mg/ml stock solution StressMarqBiosciences) and secondary antibody (goat anti-rabbit IgG AF594; 1:200 dilution of 4mg/ml stock solution Invitrogen). Hepatocyte and parasite nuclei were stained with 300 nM DAPI (Invitrogen D1306). Fluorescence signals were visualized using a Cytation imager (Biotek, Winooski, VT). The percentage of infected hepatocytes, the size of the liver-stages and the intensity of *Pf*HSP70 staining were analysed using the FIJI image analysis software package[40].

For analysis of mCherry expression in liver stages, infected hepatocytes were fixed (at day 3, 4 and 5) with 4% paraformaldehyde in 1X PBS during 1 h at room temperature. After fixation the wells were washed three times with 1X PBS and permeabilized with 20 μl of 0.5% triton in 1X PBS and then blocked with 10% of FCS in 1X PBS for 1 h. Fixed cells were washed with 1X PBS and incubated with monoclonal antibodies against *PfHSP70* (rabbit, anti-*Pfhsp70*; 1:200 dilution of 100μg/ml stock solution StressMarqBiosciences) and against mCherry (goat, anti-*mCherry* Mab Sicgen antibodies; 1:200 dilution of 3 mg/ml stock solution) for 1 h at room temperature. Subsequently, cells were rinsed 3 times with 1X PBS and incubated with the secondary antibodies Alexa Fluor®488/594-conjugated chicken anti-rabbit and anti-goat (Invitrogen Detection Technologies at 1:200). Finally, the cells were washed again three times with 1X PBS and stained with the DNA-specific dye Hoechst-33342 at a final concentration of 10μM. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and stained cells were analysed for fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa 488: 0.7 sec; Alexa 594: 0.6 sec Hoechst 0.136 sec; bright field 0.62 sec (1x gain).

Luciferase expression in liver stages was monitored daily by adding 150μl of Bright-Glo luciferase assay substrate (Promega, Madison, WI) to 150 μl of culture medium to each well and luminescence was quantified using a Synergy 2 multi-purpose plate reader (Biotek, Winooski, VT). Background was determined by measuring wells with uninfected hepatocytes.

The sensitivity of liver stage development to atovaquone determined by measurement of luminescence of infected primary human hepatocytes maintained in 96-well plates and incubated with a serial dilution of atovaquone. Atovaquone was serially diluted in DMSO and then in Williams E culture medium to reach a final DMSO concentration of 0.1%. Atovaquone was added to the 96-well cultures 3 hrs after adding 5x10^4 sporozoites to cultures of primary human hepatocytes. Medium containing drug was refreshed each day and the cultures were allowed to proceed for 4 days after which luminescence was determined as describe above.

Statistics

Data were analysed using GraphPad Prism software package 5.04 (GraphPad Software, Inc). Significance difference analyses between *WT* and the reporter line *mcherry::luc@etramp 10.3* was performed using the unpaired Student's *t*-test.

Acknowledgements

The authors are grateful to Professor Stefan Kappe for providing the *GFP-luc@eef1*α parasite line. C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). A.S. Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). Takashi Imai was, in part, supported by Uehara Memorial Foundation grant.

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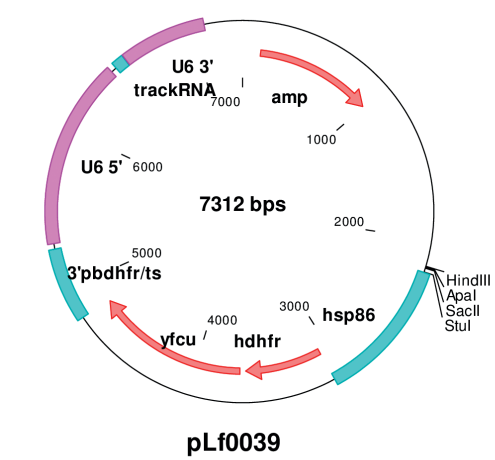
Supplementary Data



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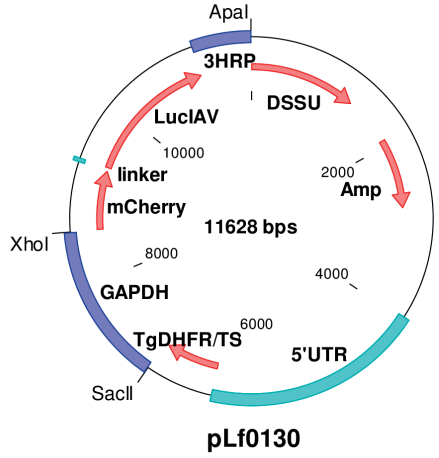

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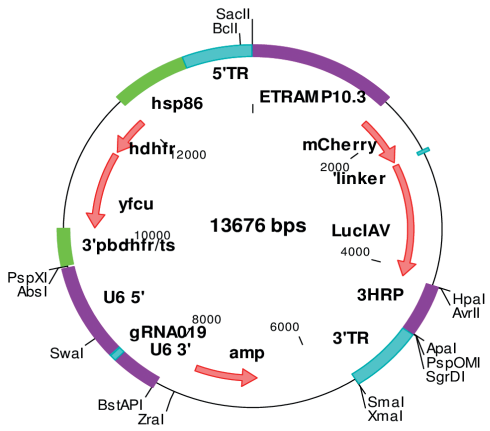

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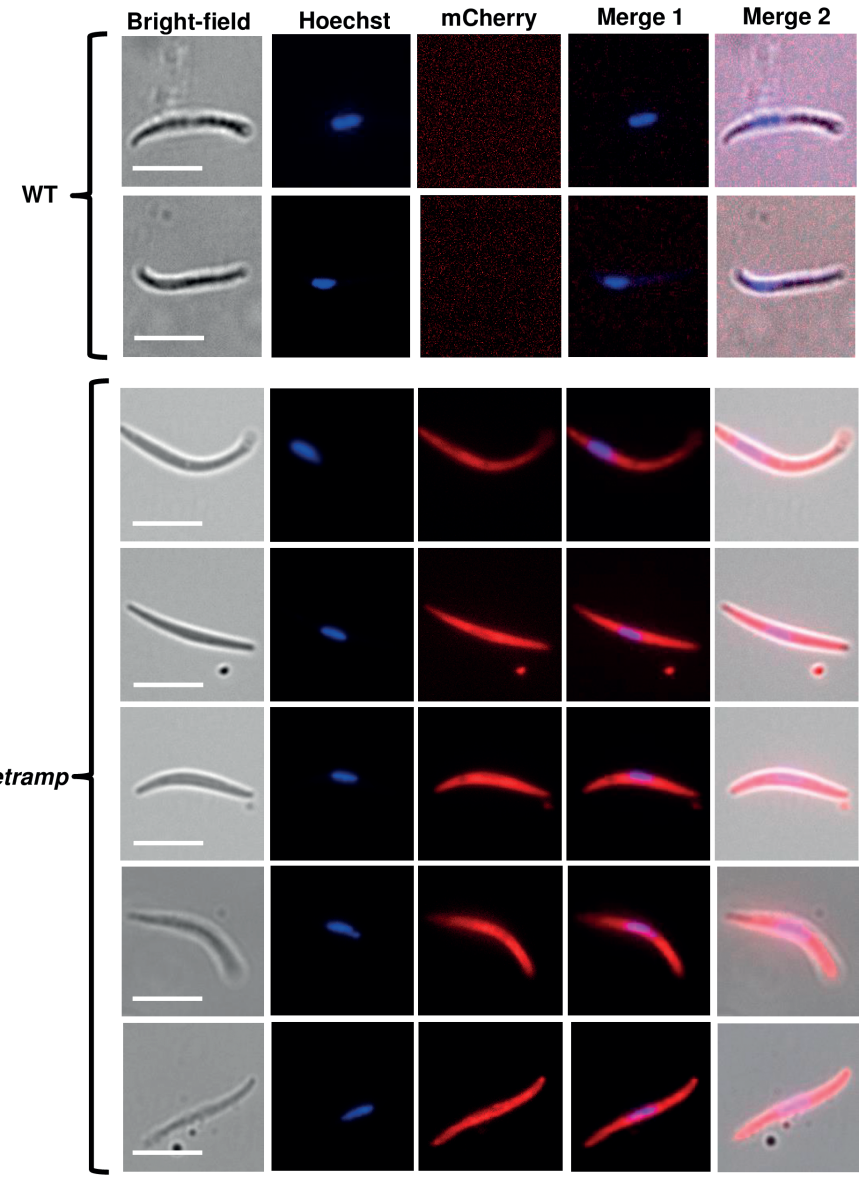
Supplementary figure 1. Maps of DNA vectors to generate the transgenic ***P. falciparum*** **line** *mCherry-luc@etramp10.3*. **A.** Vector maps of the different plasmids used to generate the *mCherry-luc@etramp* line. See Materials and Methods section for description and details of the generation of these plasmids.



Supplementary Figure 2. mCherry fluorescence of *mCherry-luc@etramp10.3* salivary glands sporozoites. Upper panel: wild type P. *falciparum* NF54 (WT) salivary gland sporozoites. Lower panel: m*Cherry-luc@etramp10.3* salivary gland sporozoites. Nuclei stained with Hoechst-33342. All pictures were recorded with standardized exposure/gain times; mCherry (red) 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 20µm.

Supplementary table 1. Gametocyte, oocyst and sporozoite production in WT and *mCherry-luc@etramp10.3* parasites

Lines	No of stage V gametocytes[1]	No. of exflagellations[2]	No. of oocyst[3]	No of sporozoites (x10^3)[3,4]
WT NF54	**range:**	**range:**	**range:**	**Range:**
	Male: 0.5-0.9	1000-1700	12-60	9-20
	Female: 0.9-1.7			
mCh-Luc@etramp	Male: 0.6	**range (SD)**	**range (SD)**	**range (SD)**
0064cl3	Female: 1.1	200-1300 (540)	20-34 (6.6)	8-10 (1.1)
	(1 exp.)	(3 exp.)	(3 exp.)	(3 exp.)

[1] Percentage stage V male and female gametocytes (per 100 red blood cells) in day 14 cultures . For WT parasites the range is given for 5 experiments
[2] Number of exflagellating male gametocytes per 10^5 red blood cells at 10-20 min after activation of day 14 gametocyte cultures). For WT parasites the range is given for 5experiments
[3] Number of oocyst per mosquito at day 9-10 after feeding (10-30 mosquitoes per exp.). For WT parasites the range is given for 5 experiments
[4] Mean number of salivary gland sporozoites per mosquito at day 21 after feeding (20-30 mosquitoes per exp.) For WT parasites the range is given for 5 experiments

Supplementary table 2. List of primers used in this study

Primer ID	Leiden code	Gene ID	Sequence	Enzymes	Product (bp)	Description
pfcsp deletion constructs						
P1	8186	PF3D7_1346800	TAATTAGGCCTGTTGCCGGCGCATACACATAAATATTTGTGTTGTAC	*StuI*/*NaeI*	794	Forward HR 1 *pfs47*
P2	8187	PF3D7_1346800	TTCCTCCGCGGGATATCCCTCCCACACTCTTGTC	*SacII*/*EcoRV*		Reverse HR 1 *pfs47*
P3	8122	PF3D7_1346800	TTATTGGGCCCGTCGACGCAATAAATTCATCGTTCAGTG	*ApaI*/*SalI*		Forward HR 2 *pfs47*
P4	8123	PF3D7_1346800	TCCTTAAGCTTCCCGGG CCACCTTGTTCACAAATACATC	*HindIII*/*SmaI*		Reverse HR 2 pfs47
P5	8126	PF3D7_1346800	TAAGTATATAATATTCAGTTGGCTTAACATTAGTCGTTTTAGAGCTAGAA			Forward gRNA019
P6	8127	PF3D7_1346800	TTCTAGCTCTAAAACGACTAATGTTAAGCCAACTGAATATTATATACTTA			Reverse gRNA019
P7	7919		GTGCCACTTTTTCAAGTTGATAACG			Sequencing gRNA019
P8	5341		GGCATCAGAGCAGATTGTAC			Sequencing gRNA019
P9		PF3D7_1016900	ATCCGCGGATAATTGTCGAAGGTTTACACATAAGGAATG	*SacII*		Forward *etramp 10.3*
P10		PF3D7_1016900	CCGGGGTACCTTTGTCGGAAATCGGATAAGAAGAAAAAAAATAATATAAAAATAAAG	*KpnI*	1703	Reverse *etramp 10.3*
Genotyping						
P11	2257		AAAGGTACCTAAAAGAAATATGAGAAC		716	Forward mCherry reporter
P12	2258		AAAAAGCTTTTTCGCCACAGGAGAAAC			Reverse mCherry reporter
P13	6767		TACGTCGCCAGTCAAGTAAC		2188	Forward 3'integration
P14	8297		CATCGAAATGCGTATTAATATGAC			Reverse 3'integration
P15	8428	PF3D7_1346800	AACTATTAAGCTCAACACAATACG		216	Forward Pfs47 ORF
P16	8429	PF3D7_1346800	CTAAATGATATGCGCTGGAATC			Reverse Pfs47 ORF

CHAPTER 6

Chimeric *Plasmodium falciparum* parasites expressing *Plasmodium vivax* circumsporozoite protein fail to produce salivary gland sporozoites

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Malaria Journal, Aug 9;17(1):288. doi: 10.1186/s12936-018-2431-1

Abstract

Background

Rodent malaria parasites where the gene encoding circumsporozoite protein (CSP) has been replaced with *csp* genes from the human malaria parasites, *Plasmodium falciparum* or *Plasmodium vivax*, are used as pre-clinical tools to evaluate CSP vaccines *in vivo*. These chimeric rodent parasites produce sporozoites in *Anopheles stephensi* mosquitoes that are capable of infecting rodent and human hepatocytes. The availability of chimeric *P. falciparum* parasites where the *pfcsp* gene has been replaced by the *pvcsp* would open up possibilities to test *P. vivax* CSP vaccines in small scale clinical trials using controlled human malaria infection (CHMI) studies.

Methods

Using CRISPR/Cas9 gene editing two chimeric *P. falciparum* parasites, were generated, where the *pfcsp* gene has been replaced by either one of the two major *pvcsp* alleles, VK210 or VK247. In addition, a *P. falciparum* parasite line that lacks CSP expression was also generated. These parasite lines have been analysed for sporozoite production in *An. stephensi* mosquitoes.

Results

The two chimeric Pf-PvCSP lines exhibit normal asexual and sexual blood stage development *in vitro* and produce sporozoite-containing oocysts in *An. stephensi* mosquitoes. Expression of the corresponding PvCSP was confirmed in oocyst-derived Pf-PvCSP sporozoites. However, most oocysts degenerate before sporozoite formation and sporozoites were not found in either the mosquito haemocoel or salivary glands. Unlike the chimeric Pf-PvCSP parasites, oocysts of *P. falciparum* parasites lacking CSP expression do not produce sporozoites.

Conclusions

Chimeric *P. falciparum* parasites expressing *P. vivax* circumsporozoite protein fail to produce salivary gland sporozoites. Combined, these studies show that while PvCSP can partially complement the function of PfCSP, species-specific features of CSP govern full sporozoite maturation and development in the two human malaria parasites.

Keywords

Malaria, *P. falciparum*, *P. vivax*, circumsporozoite protein, CSP, gene complementation

Introduction

Plasmodium sporozoites enter the blood stream through the bite of an infectious mosquito, after which they quickly migrate to the liver and invade hepatocytes. After multiplication within hepatocytes, merozoites are formed and released into the blood stream where they invade erythrocytes. Proteins of the pre-erythrocytic life cycle stages, sporozoites and liver stages, are attractive vaccine targets and are the principal components of leading malaria vaccines against the human parasites *Plasmodium falciparum* and *Plasmodium vivax* [1-4]. The target antigen of the most advanced *P. falciparum* malaria vaccine (RTS,S) is the circumsporozoite protein (CSP), the major sporozoite surface protein [5, 6] and is also an important vaccine target for *P. vivax* [7, 8]. CSP plays a critical role both in sporozoite formation and in sporozoite invasion of mosquito salivary glands and liver cells of the host [9-12]. So far, pre-erythrocytic subunit malaria vaccines, including RTS,S, have shown low to modest protective efficacy, both in the clinic and in field studies [13-16]. Efforts to increase the protective efficacy of malaria vaccines is focussed on identifying novel antigens, combining multiple antigens in a vaccine and by improving the delivery and immunogenicity of these antigens by using a variety of novel immunization platforms.

Testing the next generation of *P. falciparum* vaccines and vaccine formulations is greatly aided by the ability to vaccinate individuals and then examine vaccine efficacy by infecting them with malaria-parasites in so called controlled human malaria infections (CHMI) [17-20]. CHMI studies have increased the speed of vaccine evaluation by using well-controlled early-phase proof-of-concept clinical studies. Such studies facilitate the down-selection of vaccine candidates and identifying those most suitable for further evaluation in more expensive and difficult phase 2 and 3 trials in areas where malaria is endemic.

Although recently CHMI has also been developed for *P. vivax* [21] and has been applied to assess pre-erythrocytic vaccine candidates [22, 23], the use of *P. vivax* CHMI to rapidly screen different *P. vivax* vaccines is limited because of the lack of methods to continuously propagate *P. vivax* blood stages in culture and to produce gametocytes *in vitro* that can be used to infect mosquitoes to produce sporozoites for challenge infections [21]. Therefore, *P. vivax* CHMI is dependent on sporozoites that have been obtained from mosquitoes fed on infected patients [21]. Moreover, *P. vivax* sporozoites can produce hypnozoites, dormant forms that can persist in the liver for prolonged periods, which requires safe and effective means to clear these forms from the liver in CHMI studies [21, 24].

In preclinical evaluation of vaccines, chimeric rodent parasites expressing *P. falciparum* and *P. vivax* pre-erythrocytic antigens have been used to analyse protective immune responses induced by *P. vivax* or *P. falciparum* vaccines *in vivo* in mice. These chimeric parasites have been used to assess the protective immune responses induced by vaccination that influence sporozoite invasion of hepatocytes both *in vitro* and *in vivo* and the removal of infected hepatocytes *in vivo* [25]. For example, chimeric rodent malaria parasites have been generated where the endogenous *csp* gene has been replaced either with *pfcsp* or different *pvcsp* alleles. These chimeric parasites produce sporozoites that are infectious to rodent hepatocytes *in vivo* and human hepatocytes in culture [25].

Based on the studies with chimeric rodent parasites we reasoned that the availability of chimeric *P. falciparum* parasites that express *P. vivax* antigens would open up possibilities to analyse protective immune responses induced by vaccination using *P. vivax* antigen-based vaccines in CHMI bypassing the need for *P. vivax* parasite production and measures to ensure that *P. vivax* hypnozoites are removed. As a proof of concept two chimeric *P. falciparum* parasites were generated using CRISPR/Cas9 gene editing methodologies, where the *pfcsp* gene was replaced by one of the two major *pvcsp* alleles, VK210 and VK247 [26]. These chimeric lines, *pf-pvcsp(vk210)* and *pf-pvcsp(vk247*), had wild type-like blood stage development and produced normal numbers of oocysts. Unlike the absence of sporozoite formation in *pfcsp* deletion parasites, sporozoite formation did occur inside oocysts of both chimeric lines; however between 50 and 90% of the oocysts degenerated before sporozoite formation and no sporozoites were detected sporozoites in salivary glands. The lack of complete functional complementation was unexpected, since chimeric rodent *Plasmodium berghei* parasites expressing *Pv*CSP-VK210 and *Pv*CSP-VK247 are able to produce salivary gland sporozoites in *An. stephensi* mosquitoes that are infective to mice [27, 28]. The findings in this study and the species-specific features of CSP that may govern full maturation and development of sporozoites of the two human malaria-parasite species are discussed.

Results

Generation of two chimeric P. falciparum parasites lines expressing PvCSP-210 or PvCSP-247

Using CRISPR/Cas9 gene editing, two chimeric *P. falciparum* parasites lines were created, *pf-pvcsp*(vk210) and *pf-pvcsp*(vk247), where the *P. falciparum csp* gene has been replaced by either one of the two major *P. vivax csp* alleles, VK210 and VK247. A previously described Cas9 construct (pLf0019), containing the Cas9 expression cassette with a *blasticidin* (BSD) drug-selectable marker cassette [29], was used in combination with two sgRNA donor-DNA containing plasmids, pLf0042 and pLf0043. These constructs are used to target the *pfcsp* gene locus and as 'DNA-donor' sequences they replace *pfcsp* with either *pvcsp-vk210* or the *pvcsp-vk247* full-length gene coding sequences (**Figure 1**). The coding sequences of *pvcsp-vk210* (GenBank accession number P08677; Belem strain) and *pvcsp-vk247* (GenBank accession number M69059.1; Papua New Guinea strain) were amplified from existing plasmids P. bergheiG01-PvCSP-vk247 (pL1943) and P. bergheiG01-PvCSP-vk210 (pL1942) [27]. The two homology regions targeting *Pfcsp* (PF3D7_0304600) were amplified from genomic *P. falciparum* DNA (NF54 strain). Both constructs contain a *hdhfr-yfcu* drug-selectable marker cassette.

Transfections of *P. falciparum* NF54 parasites were performed using synchronized ring stage parasites that were transfected with ~50 µg of each circular plasmid (Cas9 and sgRNA/donor-DNA constructs; see **Figure S1**) and selection of transformed parasites containing both plasmids (Cas9 and sgRNA/donor-DNA constructs) was performed by

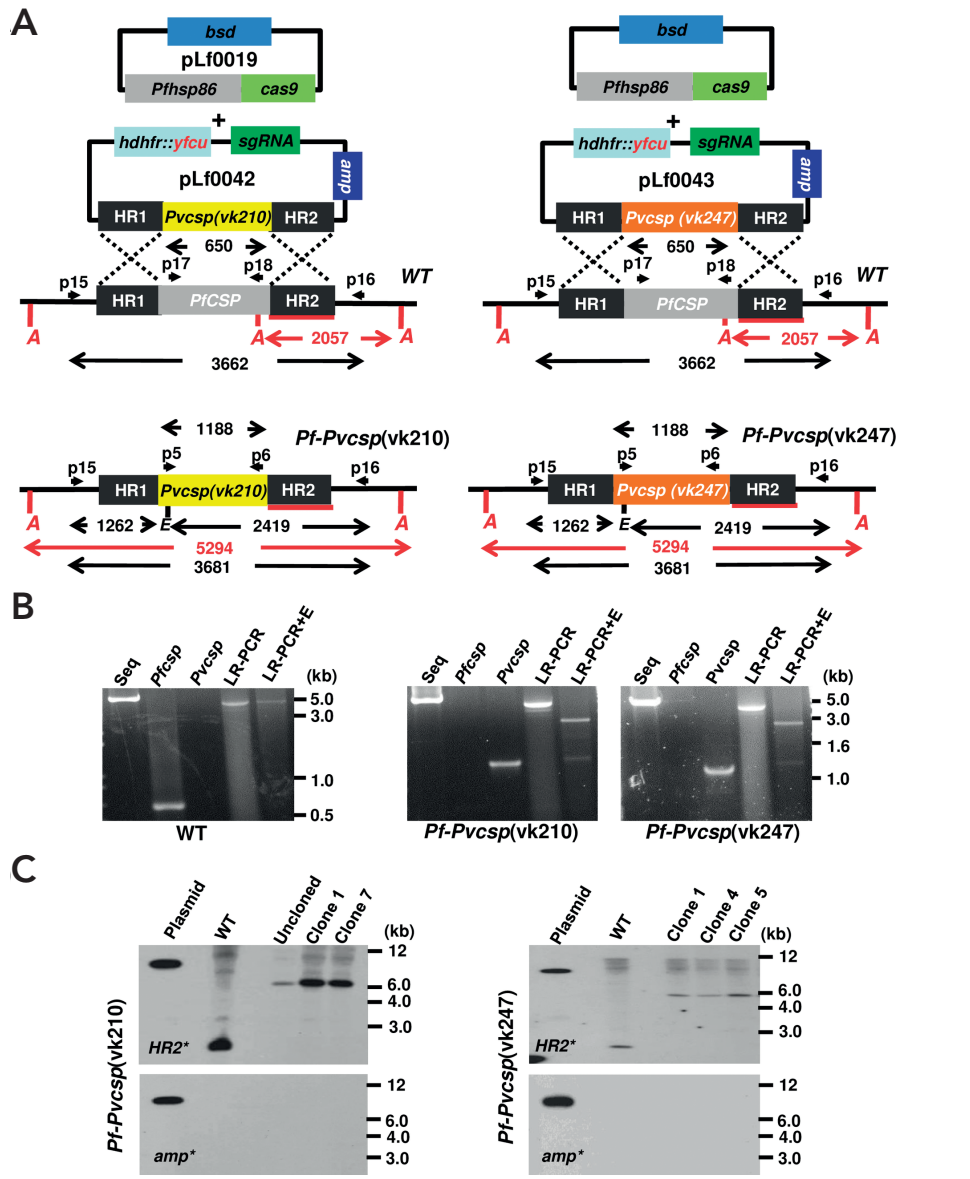


Figure 1. Generation and genotyping of two chimeric *P. falciparum* parasites *(Pf-pvcsp).* **A** Two *Pf-pvcsp* parasite lines were generated using CRISPR/Cas9 methodology. The coding sequence (CDS) of *Pfcsp* gene was replaced by insertion of the *Pvcsp*(vk210) and *Pvcsp*(vk247) CDS using donor-DNA plasmids pLf0042 and pLf0043. A schematic representation of the *Pfcsp* locus before and after insertion of the construct showing the location of the restriction sites (*A: Ava*II, *E: EcoR*V), sizes (in bp) of restriction fragments (red for Southern blot analysis), location of primers (p), PCR amplicons and sizes (in bp) of the fragments (in black (**B, C**). HR1, HR2: *Pfcsp* homology (targeting) regions. The figure is not shown to scale. Primer sequences can be found in **Additional file 7**. **B**. Diagnostic PCR and long-range PCR (LR-PCR) confirming the correct integration of the *Pvcsp* CDS into the *PfCSP* locus. Diagnostic PCR: *Pfcsp* open reading frame (lane 2; primers p17/p18); *Pvcsp* open reading frame (lane 3; primers p5/p6); *P. falciparum sequestrin* gene as a control gene (lane 1; primers p22/ ▸

p23). LR-PCR: products were run undigested or digested with *EcoR*V (LR-PCR+E) in order to confirm double cross-over recombination. LR-PCR (lane 4) of cloned parasites of *Pf-pvcsp*(vk210)(cl7; primers p15/p16), *Pf-pvcsp*(vk247)(cl5; primers p15/p16) and WT. LR-PCR fragments digested with *EcoR*V (lane 5) for confirmation of double cross-over integration. **C.** Southern blot analysis of *Ava*II restricted DNA of WT and chimeric *Pf-Pvcsp* parasites confirms the specific integration of the *Pvcsp* genes into the *pfcsp* gene locus. DNA was hybridized with a probe targeting the homology region 2 of *pfcsp* (upper panels; HR2; primers p3/p4; see **A**). In addition, to show absence of donor-DNA plasmid and single cross-over events, DNA was hybridized with a probe for the ampicillin gene (lower panels; intermediate donor-DNA plasmid pLf0040 digested with *Aat*II and *Pvu*I). The hybridization pattern observed with the HR2 probe identified the expected different-sized DNA fragments in WT and *pf-pvcsp* parasites (2057 bp and 5294 bp).

applying 'double' positive selection with the drugs WR99210 and BSD, until parasites were detectable by thin blood-smear analysis (between day 14 to 26 post transfection). Subsequently, parasites were cultured for 2-4 days without drugs, followed by applying negative selection to eliminate parasites that retained the transfection constructs (i.e. donor-DNA) as episomal plasmids and to enrich for transfected parasites in which the donor-DNA construct has integrated into the parasite genome. Genotyping of selected parasite populations by long-range PCR revealed that both *pvcsp*(vk210) and *pvcsp*(vk247) cassettes had integrated into the *P. falciparum* genomes (**Figure 1B**) and Southern blot analysis of cloned lines confirmed correct integration of the constructs (**Figure 1C**). Phenotype analyses, as described below, were performed using *pf-pvcsp*(vk210) clone 7 and *pf-pvcsp*(vk247) clone 5. Sequence analysis of the long-range PCR products confirmed the correct sequence of *pvcsp* genes and replacement of the *pfcsp* gene in both *pf-pvcsp* lines (See **Figure S2**).

Pf-PvCSP210 and Pf-PvCSP210 parasites form oocysts but salivary gland sporozoites are absent in An. stephensi mosquitoes

The growth of asexual blood stages in cultures and gametocyte production of both *pf-pvcsp* lines was comparable to that of the parental *P. falciparum* NF54 wild type (WT) parasite strain (see **Figure S3, Table 1**). Gametocyte cultures of the *Pf-PvCSP* lines produced WT-like numbers of mature, stage V, gametocytes of both sexes. Mature male gametocytes of both lines underwent exflagellation upon activation and were able to form male gametes (**Table 1**).

Anopheles stephensi mosquitoes were fed with either WT gametocytes or gametocytes of the *pf-pvcsp* lines using the standard membrane feeding assay and the number of oocysts in mosquito midguts was determined at day 10 post infection and the presence of sporozoites in the haemocoel and salivary glands was analysed at day 14 and day 21. Dissection of WT and *pf-pvcsp* infected mosquitoes revealed that all lines produced comparable numbers of oocysts; however, no sporozoites were detected in salivary glands in mosquitoes infected with either of the *pf-pvcsp* lines (**Table 1**)**.** Moreover, no sporozoites from the *pf-pvcsp* lines were found in haemocoel fluid after mosquito dissection; in

Table 1. Gametocyte, oocyst and sporozoite production in WT, *pf-pvcsp(vk210)*, *pf-pvcsp(vk247)* and *Pf*Δ*csp*

Lines	No of gametocytes Stage V male/female mean (SD) [1]	No. of exflagellation mean (SD) [2]	No. of oocyst mean (range)[3]	No of sporozoites[4]
WT	m: 0.6 (0.2) f: 1.3(0.3)	1436 (191)	28.2 (13-54)	9K-20K
NF54 (n=4)	(3 exp.)	(3 exp.)	(4 exp)	(4 exp)
***pf-pvcs*(vk210)**				
0050cl7 (n=5)	m: 0.6 ; f: 1.1 (1 exp.)	2030 (916) (3 exp.)	44.2 (30-63) (5 exp)	Negative
0050cl2 (n=1)	ND	ND	9.1 (1 exp.)	Negative
***pf-pvcs*(vk247)**				
0041cl5 (n=3)	m: 0.8; f:1.5 (1 exp.)	460 (34) (3 exp.)	23.1 (17-40) (3 exp.)	Negative
Pf*Δ*csp				
0113cl1 (n=1)	ND	875 (1exp.)	4.6	Negative
0113cl3 (n=2)	m: 0.6 f: 1.1 (1 exp.)	1010 (388) (3 exp.)	2.2 and 3.2 (2 exp.)	Negative

[1] Mean percentage of stage V male (m) and female (f) gametocytes (per 100 red blood cells) in day 14 cultures in 2-7 experiments (exp.)
[2] Mean number of exflagellating male gametocytes (per 10^5 red blood cells) at 10-20 min after activation of day 14 gametocyte cultures (s.d.: standard deviation)
[3] Mean number of oocyst per mosquito at day 9-10 after feeding. Range corresponds to the mean number of retorts in multiple experiments (1-5 exp. per line; 10-30 mosquitoes per exp.)
[4] Mean number of salivary gland sporozoites per mosquito at day 21 after feeding. Range corresponds to the mean number of sporozoites in multiple experiments (1-5 exp. per line; 20-30 mosquitoes per exp.)

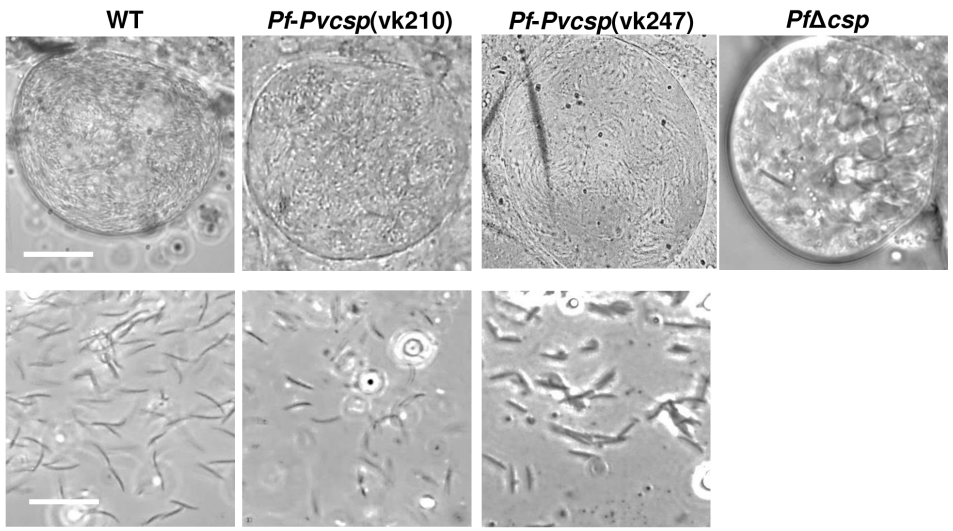
contrast, when WT infected mosquitoes were dissected many sporozoites were observed in haemocoel fluid.

These observations indicate that the chimeric parasites expressing PvCSP are unable to produce sporozoites that are competent in invading salivary glands.

pf-pvcsp lines produce sporozoites in oocysts but most oocysts degenerate before sporozoite formation and release of sporozoites

For two *Plasmodium* species it has been shown that *csp* deletion mutants can form oocysts; however by light-microscopy, these oocysts are highly vacuolated and do not show signs of sporozoite formation [30, 31]. Therefore, oocyst development of *pf-pvcsp* was analysed in greater detail by light microscopy and *Pv*CSP expression was examined by immunofluorescence microscopy. After feeding with both *pf-pvcsp* lines, oocysts were readily detected at day 10, in which sporozoite formation occurred (**Figure 2A**). However, analysis of oocysts between day 10 and 14 showed that most oocysts started to degenerate before full maturation as shown by the absence of clear sporozoite formation and the presence of large vacuoles in the cytoplasm of maturing oocysts (see Figure S4). In WT-infected mosquitoes, 30% of the oocysts were characterized as degenerate at day

A



B

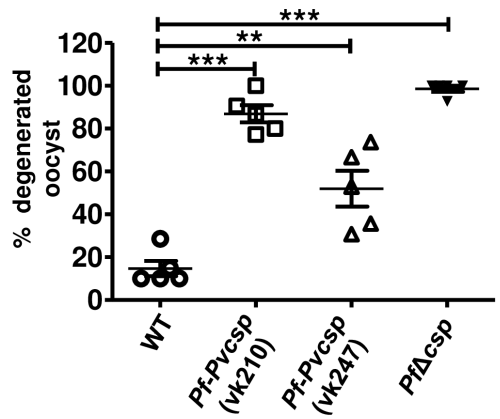


Figure 2. Oocyst and sporozoite formation of two chimeric *P. falciparum* parasite lines *(pf-pvcsp)* and a PfCSP knockout line *(PfΔcsp)*. **A**. Light microscope pictures of oocysts at day 10 after feeding gametocytes to *Anopheles stephensi* mosquitoes. Upper panel *pf-pvcsp* and wild type *P. falciparum* (WT) oocyst in which sporozoite formation occurs (see Additional file 3 for pictures of *pf-pvcsp* oocyst that degenerate before sporozoite formation). No sporozoite formation was observed in *PfΔcsp* oocysts. Scale bar, 20µm. Lower panel: free *pf-pvcsp* sporozoites that are released from oocysts by application of force to oocysts in dissected midguts. Scale bar, 20µm. **B.** Percentage of degenerated oocyst in *An. stephensi* mosquitoes (n=5) at day 10 after feeding (**p=0,0035, ***p=<0,0001; unpaired T-test).

10, whereas in *Pf-Pv*CSP(vk210) and *Pf-Pv*CSP(vk247) infected mosquitoes 52% and 87% degenerate oocysts were countedy (**Figure 2B**). No spontaneous release of sporozoites from mature *pf-pvcsp* oocysts was detected and no sporozoites could be found free in the haemocoel or in salivary glands, even up to day 21 after feeding. Only through the application of force to oocysts in dissected midguts could mechanically liberated oocyst-derived *pf-pvcsp* sporozoites be recovered (**Figure 2A**).

Chimeric sporozoites of both pf-pvcsp(vk210) and pf-pvcsp(vk247) express PvCSP

To analyse expression of PvCSP in *pf-pvcsp* sporozoites, oocyst-derived sporozoites were collected by differential centrifugation of extracted, mechanically crushed, infected midguts. These sporozoites were stained with antibodies specific for *Pv*CSP-VK210, *Pv*CSP-VK247 and PfCSP. Sporozoites of *pf-pvcsp(vk247)* and *pf-pvcsp(vk210)* only reacted with their cognate anti-PvCSP-VK247 or anti-PvCSP-VK210 antibodies and WT sporozoites only with anti-PfCSP antibodies (**Figure 3; Figure S4**). These results indicate that the corresponding PvCSP is expressed in developing oocysts and oocyst-derived sporozoites of the *pf-pvcsp* lines and that the failure of formation of fully competent sporozoites is not due to the absence of PvCSP expression.

A P. falciparum NF54 mutant that lacks expression of PfCSP forms oocysts but sporozoite formation is absent

To investigate whether the formation of sporozoites in chimeric *pf-pvcsp* oocysts was due to partial complementation of PfCSP by PvCSP, a *P. falciparum* mutant lacking CSP expression was generated. This mutant (*PfΔcsp*) was generated by disrupting the *pfcsp* gene by CRISPR/Cas9 gene editing using a construct (pL0083) that contained an mCherry reporter cassette (under control of the constitutive *gapdh* promoter; see **Figure S5A**) flanked by *pfcsp* targeting sequences. This construct is designed to replace the *pfcsp* gene with the mCherry-expression cassette and contains a *hdhfr-yfcu* drug-selectable marker cassette. Transfection of *P. falciparum* NF54 parasites was performed by spontaneous plasmid uptake from plasmid-loaded red blood cells cultured under static conditions. Uninfected RBC were mixed with 50 µg of two sgRNA/Cas9 constructs (pLf0071 and pLf0072) and 50 µg of the donor-DNA construct (pLf0083). After electroporation, these uninfected cells were mixed with iRBC containing *P. falciparum* NF54 parasites and selection of transformed parasites was performed with the drug WR99210 during a period of 6 days. Subsequently the drug was removed and parasites were harvested at 0.6-0.8% parasitaemia for mCherry fluorescence microscopy analysis to determine the ratio of WT and mutant parasites expressing mCherry. Parasites were collected from cultures that contained >80% mCherry-positive parasites (at a 4-10% parasitaemia). Genotyping of selected and cloned mCherry-positive parasites by diagnostic PCR and Southern blot analysis showed integration of the mCherry-expression cassette into the *pfcsp* gene (see **Figure S5B and C**).

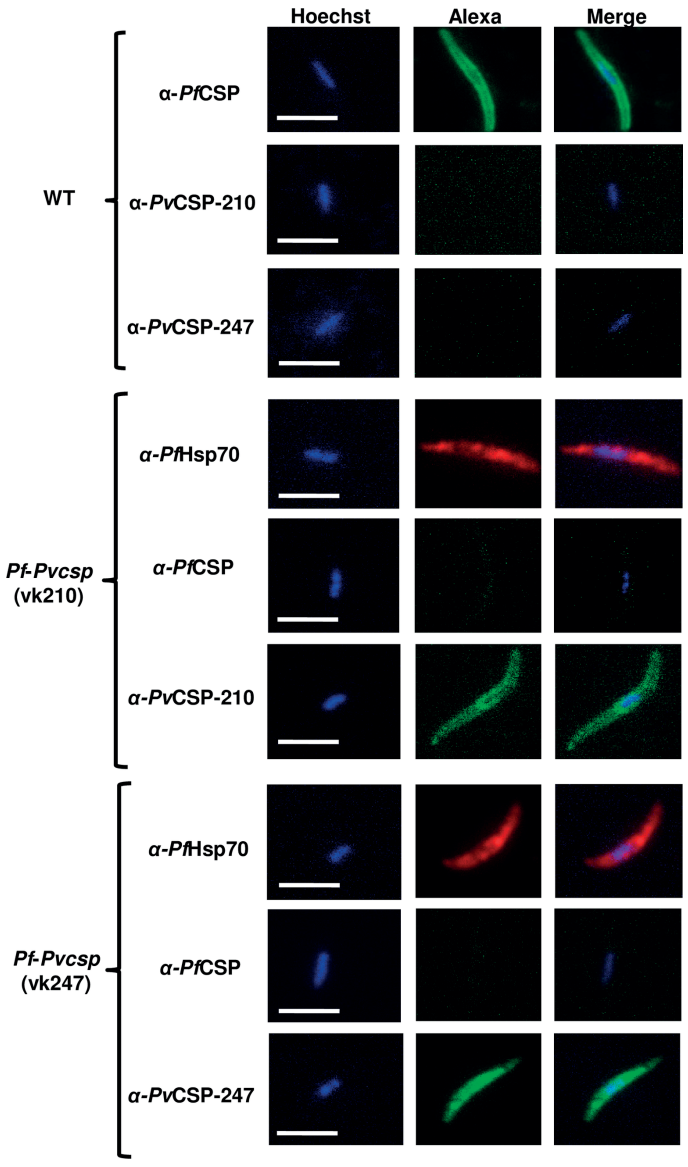


Figure 3. PvCSP(VK210) and PvCSP(VK247) expression in oocyst-derived sporozoites of two chimeric *P. falciparum* parasite lines *(pf-pvcsp)*. Immunofluorescence analyses of wild type *P. falciparum* (WT) sporozoites and oocyst-derived *pf-pvcsp* sporozoites. Fixed sporozoites were labelled with mouse anti-PvCSP-VK210 mAb, anti-PvCSP-VK247mAb and mouse anti-PfCSP antibodies. As a control an antibody against PfHSP70 was used. Secondary conjugated antibodies used: anti-IgG Alexa Fluor® 488 (green) or anti-IgG Alexa Fluor ® 594 (red). Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/gain times; Alexa Fluor® 488 (green) 0.7 s; anti-IgG Alexa Fluor ® 594 (red) 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7µm.

The growth of asexual blood stages in cultures and gametocyte production of the *Pf*Δ*csp* line was comparable to that of WT *P. falciparum* NF54 parasites (see **Figure S3; Table 1**). Gametocyte cultures of the *Pf*Δ*csp* lines produced WT-like numbers of mature, stage V, gametocytes of both sexes. Mature male gametocytes of both lines underwent exflagellation upon activation and were able to form male gametes (**Table 1**). *Anopheles stephensi* mosquitoes were fed with WT gametocytes and *Pf*Δ*csp* gametocytes using standard membrane feeding. The number of oocysts was determined at day 10 post feeding and the presence of sporozoites in salivary glands analysed at day 14 and 21 after feeding. *Pf*Δ*csp* parasites were able to produce oocysts in the mosquitoes but no sporozoites were detected in salivary glands after feeding (**Table 1**). Moreover, sporozoite formation was not detected in oocysts (3 exp.; 30 mosquitoes per experiment). All oocysts degenerated before full maturation as shown both by the absence of sporozoite formation and the presence of large vacuoles in the cytoplasm of the oocysts (see **Figure 2; Figure S6**). This oocyst phenotype is comparable to the phenotype observed in *P. berghei* and *Plasmodium knowlesi* mutants lacking CSP expression [30, 31]. The absence of sporozoite formation in *Pf*Δ*csp* oocysts is in support of partial complementation of PfCSP by PvCSP in the *pf-pvcsp* lines where sporozoites are formed in a fraction of the oocysts.

Heterologous CSP replacement and expression in different Plasmodium species

Several rodent malaria parasite mutants have been generated in which their endogenous *csp* gene has been replaced by a *csp* gene from another *Plasmodium* species. In most of these mutants the heterologous CSP protein can complement the function of endogenous CSP **(Table 2).** In both *Plasmodium yoelii* and *P. berghei*, *P. falciparum* CSP can replace and functionally complement rodent parasite CSP. In addition, multiple chimeric *P. berghei* lines have been generated that express *P. vivax* CSP alleles (**Table 2**) and these lines produce salivary gland sporozoites that are capable of infecting mice. In *P. berghei* only replacement of PbCSP with CSP of the avian malaria parasite *Plasmodium gallinaceum* did not lead to full complementation and *pb-pgcsp* parasites produced strongly reduced numbers of salivary gland sporozoites, which are not infective to mice [32]. Recently, the generation of two chimeric *pb-pvcsp* lines, expressing the *pvcsp* (*vk210/vk247*) genes was reported [27]. The *pvcsp-210* and *pvcsp247* genes used to generate the chimeric *pf-pvcsp* lines described in this study were amplified from the constructs used to generate the rodent *pb-pvcsp* lines. The *pb-pvcsp* lines produced salivary gland sporozoites in *An. stephensi* mosquitoes that were fully infectious to mice and these lines have been used to analyse protective immunity induced in mice by vaccines that target PvCSP [27]. Oocyst formation and sporozoite production of *pb-pvcsp(vk210)* was comparable to that of WT parasites but sporozoite production of *pb-pvcsp(vk247)* was reduced (Table 2). Oocyst formation and sporozoite production of these lines was analysed in more detail and confirmed the WT-like formation of oocysts and sporozoites in *pb-pvcsp(vk210)* (see **Figure S7**). The number of salivary gland sporozoites of *pb-pvcsp(vk247)* was however

Table 2. Parasite lines of different *Plasmodium* species expressing heterologous or mutated CSP and mutants lacking CSP expression

Plasmodium species	CSP[1]	Oocyst no. [2]	Salivary gland sporozoite no. [2]	Reference; RMgmDB ID[3]	Remarks (mutation, sporozoite phenotype)
Chimeric CSP parasite lines					
P. berghei	*Py* CSP	WT	WT	[32]; 75	
P. berghei	*Pf* CSP	WT	Reduced (90%)	[52]; 69	
P. berghei	*Pf* CSP	WT	Reduced (90%)	342	
P. berghei	*Pf* CSP	WT	WT	[53]; 4110	
P. berghei	*Pf* CSP	WT	WT	[54]; 4135	
P. yoelii	*Pf* CSP	WT	WT	[55]; 1442	
P. berghei	*Pv* CSP-210	WT	WT	[27]; 4136	
P. berghei	*Pv* CSP-247	WT	WT	[28]; 1443	
P. berghei	*Pv* CSP-247	WT	Reduced (30%)	[27]; 4137	
P. berghei	*Pg* CSP	WT	Absent	[32]; 74	WT oocyst sporozoite formation
P. falciparum	*Pv* CSP-210	WT	Absent	This study	Sporozoite formation in fraction of oocysts
P. falciparum	*Pv* CSP-247	WT	Absent	This study	Sporozoite formation in fraction of oocysts
Knock-out CSP parasite lines					
P. berghei	-	WT	Absent	[30]; 9	No sporozoite formation
P. knowlesi	-	WT	Absent	[31]	No sporozoite formation
P. falciparum	-	WT	Absent	This study	No sporozoite formation
Mutated CSP parasite lines (with a sporozoite production phenotype)					
P. berghei	Mut. Pb CSP	WT	Absent	[33]; 72	PbCSP with truncated 3'UTR; reduced sporozoite formation in oocysts
P. berghei	Mut. Pb CSP	WT	Absent	[38]; 73	Mutations of the C-terminal GPI-anchor. No sporozoite formation in oocysts
P. berghei	Mut. Pb CSP	WT	Absent	[34]; 1148	PbCSP lacking repeat region; reduced sporozoite formation in oocysts; no midgut/salivary gland sporozoites
P. berghei	Mut. Pb CSP	WT	Absent	[34]; 1149	PbCSP lacking repeat region and NH2 terminus; no sporozoite formation in oocysts;
P. berghei	*Pb*/*Pg* CSP	WT	Absent	[56]; 770	*pbcsp* replaced by *pgcsp* with repeat region of *pbcsp*

[1] Expression of heterologous CSP or mutated CSP
[2] Oocyst numbers and salivary gland sporozoite numbers in infected *An. stehensi* mosquitoes compared to wild type (WT) infected mosquitoes. WT = numbers in the same range as WT-infected mosquitoes
[3] Mutant ID in the RMgmDB database: www.pberghei.eu

significantly reduced (p<0.0001***) compared to WT or *pb-pvcsp(vk210)* parasites (see **Figure S7**). By analysis of oocysts of both lines at day 14 post feeding, we observed increased numbers of degenerated oocysts in the *pb-pvcsp(vk247),* a phenotype that was similar to oocysts of the *pf-pvcsp* lines, i.e. absence of sporozoite formation and vacuolated oocyst morphology (see Additional file 6). These observations indicate that the differences in numbers of salivary gland sporozoites between the *P. berghei* lines results from a better ability of *P. vivax* CSP(VK210) to complement PbCSP function than PvCSP(VK247).

Discussion

Chimeric *P. falciparum* parasites where the *csp* gene has been replaced with coding sequences of *P. vivax csp*, either *Pvcsp*(vk210) or *Pvcsp*(vk247), do not form salivary gland sporozoites. These observations indicate that *Pv*CSP cannot functionally complement *Pf*CSP. Although *Pv*CSP-expressing sporozoites are formed within oocysts of both chimeric lines, most oocysts degenerate before sporozoite formation and no sporozoites are released from oocysts resulting in the lack of sporozoites in the haemocoel or in salivary glands. The inability of *P. vivax* CSP to functionally complement *P. falciparum* CSP is unexpected as studies in the rodent parasite *P. berghei* have shown that the *P. berghei* CSP can be functionally replaced by CSP from different *Plasmodium* species, including the human *Plasmodium* species, *P. vivax* and *P. falciparum* (Table 2). Chimeric *pb-pvcsp* sporozoites expressing the same two PvCSP alleles VK210 and VK247, which were used in this study, are able to invade *An. stephensi* salivary glands and are infectious to mice.

CSP is a multifunctional protein that has an essential role in the formation of sporozoites inside oocysts as well as in sporozoite release, motility and host-cell invasion [9-12]. Mutants of *P. berghei* and the primate parasite *P. knowlesi* lacking CSP expression do form oocysts but sporozoite formation inside oocysts is absent [30, 31]. Maturing oocysts of these *csp*-deletion mutants are highly vacuolated and have no signs of sporozoite formation that could be detected by light microscopy. In addition, highly vacuolated oocysts and absence of sporozoite formation were observed in mosquitoes fed with the *P. falciparum* mutant lacking CSP, *Pf*Δ*csp* generated in this study. These observations confirm the essential role of CSP early in the formation of *Plasmodium* sporozoites and in oocyst maturation.

In contrast to the *Pf*Δ*csp* parasites, where no sporozoite formation was detected in maturing oocysts, we observed *Pf-Pvcsp* oocysts with sporozoite formation and we were able to obtain oocyst-derived sporozoites of both chimeric lines. These sporozoites expressed *Pv*CSP as shown by immunofluorescence analysis with antibodies specific for either *Pv*CSP VK210 or VK247. These observations indicate that the *Pv*CSP proteins can be used to initiate sporozoite formation in *P. falciparum* oocysts but are unable to fully complement the function of *Pf*CSP in oocyst maturation and sporozoite development. Despite the formation of typical elongated sporozoites in some oocysts of *Pf-Pvcsp* fed mosquitoes, most oocysts exhibit a vacuolated morphology and degenerate before sporozoite formation. Oocyst degeneration was clearly visible from day 10 onwards and

between day 10 and 21 no increase in oocysts with sporozoite formation was observed, indicating that the absence of sporozoite formation at day 10 is not the result of a delayed maturation of the oocysts. In addition, no evidence was found for spontaneous release of sporozoites of the oocysts that contained sporozoites and we did not observe haemocoel or salivary gland sporozoites in *Pf-Pvcsp* infected mosquitoes up to day 21 post feeding. Free sporozoites were only observed when oocysts were ruptured by applying mechanical forces on these oocysts.

It seems unlikely that the failure of PvCSP to functionally complement PfCSP is due to incorrect expression of the PvCSP proteins in the *pf-pvcsp* lines. The same *pvcsp* genes as used for successful complementation of CSP in *P. berghei* [27] were used to replace *P. falciparum csp* and the *pvcsp* genes were amplified from the same plasmids that were used for generation of the *pb-pvcsp* lines. In addition, the *pvcsp* gene coding sequence in the genome of the *pf-pvcsp* lines was placed under control of the endogenous *pfcsp* promoter and transcriptional terminator sequences to ensure correct timing and level of CSP expression. It has been shown that the 3' untranslated region (3'-UTR) of *P. berghei csp* plays an important role in accurate CSP expression as truncation of *pbcsp* 3'-UTR results in reduced CSP expression, reduced oocyst sporozoite formation and degeneration of oocysts [33].

The inability of PvCSP to replace the PfCSP function in the chimeric *pf-pvcsp* lines is therefore most likely due to sequence differences between these *csp* genes that lead to structural differences of the CSP proteins, which may affect interactions with other parasite proteins that are essential for proper sporozoite formation. These can be protein interactions that influence correct transport of CSP from within the oocyst-cytoplasm to the membrane of developing sporozoites or interactions that affect its localization or maintenance on sporozoites [9-12, 34]. Mutational analyses of *P. berghei* CSP have shown that different regions/sequence motifs of CSP are involved in correct formation of sporozoites in oocysts (**Table 2**). The overall structure of CSP of different *Plasmodium* species is conserved. CSP is a GPI anchored protein that has a central amino acid repeat region, the sequence and number of repeats varies across *Plasmodium* species. These repeats are flanked by two conserved domains; region I at the N terminus of the repeats, and the thrombospondin repeat (TSR) domain C-terminal to the repeats [9]. The repeat regions of PfCSPs consist of predominantly NANP repeats, which differs in length between individual *P. falciparum* strains [35, 36]. The repeat region of CSP of two major strains of *P. vivax*,VK210 and VK247 are different from PfCSPs and these consists of 10-11 copies of GDRA(A/D)GQPA or ANGA (G/D)(N/D)QPG in CSP-VK210 and CSP-VK247, respectively[37]. The GPI-anchor and the repeat region have been shown to play an essential role in correct sporozoite formation in *P. berghei* oocysts (**Table 2**). Mutant parasites expressing CSP without GPI-anchor, or with a mutated GPI-anchor, fail to produce sporozoites and the phenotype is similar to mutants that lack CSP expression, i.e. complete absence of sporozoite formation [38]. The repeat region of CSP appears to play a critical role in the formation of *P. berghei* sporozoites. *P. berghei* parasites expressing mutated CSP lacking only the repeat region

are affected in the later stages of sporozoite formation [34], a phenotype that more closely resembles the phenotype of the *pf-pvcsp* lines. It has been proposed that the CSP repeats play a structural role and their absence may result in misfolding of CSP and this could affect the interaction of CSP with other sporozoite proteins, which are required for final oocyst and sporozoite maturation [34]. However, *P. berghei* parasites that lack the N-terminal portion of CSP, but retain the signal sequence, the repeat region and the C'-terminal region of the protein, can still produce salivary gland sporozoites[39] The phenotype of chimeric *P. falciparum* expressing PvCSP might therefore be explained by a disturbed interaction of PvCSP with other *P. falciparum* proteins, interactions that are mediated by the repeat sequences and which are necessary for complete maturation and release of oocyst sporozoites. In contrast, it would appear that both PvCSP and PfCSP are able to interact with these CSP interacting proteins in *P. berghei*. *Plasmodium berghei* parasites where PbCSP has been replaced with either PvCSP(VK210) or PvCSP(VK247) produced infectious salivary gland sporozoites, although parasites expressing PvCSP(VK247) produced significantly less salivary gland sporozoites than either WT or PvCSP(VK210)-expressing parasites. Mosquitoes infected with *pb-pvcsp(vk247)* contained degenerate oocysts with a vacuolated morphology that resembled *pf-pvcsp* parasites. These observations indicate that *Pv*CSP(VK247) is less effective in complementing *P. berghei* CSP function in sporozoite maturation and release from oocysts. However, the *pb-pvcsp(vk247)* sporozoites that had invaded the salivary glands were not affected in their ability to infect mice.

Further studies using parasites expressing chimeric CSP molecules, comprised of different domains of PfCSP and PvCSP, are required to reveal which CSP domains are essential for sporozoite maturation and release and can explain the failure of PfCSP complementation by PvCSP. The inability of *pf-pvcsp* lines to produce salivary gland sporozoites means that these lines cannot be used for CHMI studies.

Conclusions

Chimeric *P. falciparum* parasites expressing *P. vivax* circumsporozoite protein fail to produce salivary gland sporozoites. While PvCSP-expressing sporozoites are formed within oocysts, most oocysts degenerate before sporozoite formation, no sporozoites are released from oocysts and results in the absence of sporozoites in either the mosquito haemocoel or salivary glands. Combined, these observations show that while PvCSP can partially complement the function of PfCSP, species-specific features of CSP govern full sporozoite maturation and development in the two human malaria parasites.

The inability of *P. vivax* CSP to functionally complement *P. falciparum* CSP is unexpected as studies in the rodent parasite *P. berghei* have shown that the *P. berghei* CSP can be functionally replaced by CSP from different *Plasmodium* species, including the human *Plasmodium* species, *P. vivax* and *P. falciparum*. It seems unlikely that the failure of PvCSP to functionally complement PfCSP is due to incorrect expression of the PvCSP proteins, as

the same *pvcsp* genes were used that were able to successfully complement the function of CSP in *P. berghei*. Further studies on the role of different CSP elements from different *Plasmodium* species and their potential interactions with other *Plasmodium* proteins may not only reveal the species-specific mechanisms that govern sporozoite formation and function but may also provide essential information that can be used to create human infectious chimeric *pf-pvcsp* sporozoites.

Materials and methods

Plasmodium falciparum and Plasmodium berghei parasites and in vitro cultivation of P. falciparum blood stages

Plasmodium falciparum parasites from the NF54 strain[40] were obtained from the Radboud University Medical Center (Nijmegen, The Netherlands). Parasites were cultured following the standard conditions in RPMI-1640 culture medium supplemented with L-glutamine and 25mM HEPES (Gibco Life Technologies), 50 mg/L hypoxanthine (Sigma). Culture medium was supplemented with 10% human serum and 0.225% $NaHCO_3$. Parasites were cultured at a 5% haematocrit under 4% O_2, 3% CO_2 and 93% N_2 gas-conditions at 75 rpm at 37°C in a semi-automated culture system in 10ml flasks (Infers HT Multitron and Watson Marlow 520U). Fresh human serum and human red blood cells (RBC) were obtained from the Dutch National Blood Bank (Sanquin Amsterdam, the Netherlands; permission granted from donors for the use of blood products for malaria research and microbiology test for safety). RBC of different donors were pooled every two weeks, washed twice in serum free RPMI-1640 and suspended in complete culture medium to 50% haematocrit. Human serum of different donors were pooled every 4-6 months and stored at -20°C until required.

In addition, *P. falciparum* gametocytes cultures were generated using standard culture conditions (see above) with some modifications [41]. Briefly, parasites from asexual stage cultures were diluted to a final parasitaemia of 0.5% and cultures were followed during 14 days without refreshing RBC. After 9 days these cultures were treated with 50mM of N-acetyl-D-glucosamine (Sigma) to kill asexual stages and to enrich for gametocytes. At day 14 the cultures were analysed for mature, stage V, gametocytes.

Four different mutant lines of the rodent parasite *P. berghei* were used that have been previously reported. i) A transgenic reference line of *P. berghei* ANKA, expressing the fusion protein GFP-Luciferase (line 676m1cl1; *PbΔp230p*; RMgm-29; www.p*berghei*.eu)[42]; ii) A mutant that expresses *P. vivax* CSP (VK210 allele). In this mutant the *pbcsp* gene has been replaced with the *pvcsp-vk210* gene (line 2196cl1; RMgm-4136; www.p*berghei*.eu); [27]; iii) A mutant that expresses *Pv*CSP (VK247 allele). In this mutant the *pbcsp* gene has been replaced with the *pvcsp-vk247* gene (line 2199cl1; RMgm-4137; www.p*berghei*.eu; [27];

Generation and selection of the chimeric lines pf-pvcsp(vk210) and pf-pvcsp(vk247)

In order to create *pf-pvcsp(vk210)* and *pf-pvcsp(vk247)*, the previously described pLf0019 construct, containing the *cas9* gene was used [29] and 2 different sgRNA-donor DNA containing plasmids, pLf0042 (targeting *Pf*CSP and containing the *pvcsp-vk210* gene) and pLf0043 (containing the *pvcsp-vk247*). The pLf0019 construct contains a *blasticidin* (BSD) drug-selectable marker cassette and both sgRNA-donor DNA constructs (pLf0042 and pLf0043) contain a h*dhfr* drug-selectable marker cassette for selection with WR99210. To generate pLf0042 and pLf0043, plasmid pLf0033 (see **Figure S1**) was modified by introducing two homology regions targeting *Pfcsp* (PF3D7_0304600). Homology region 1 (HR1) was amplified using primers P1/P2 and homology region 2 (HR2) with P3/P4 from *P. falciparum* NF54 genomic DNA (see **Table S1** for primer details). HR1 was cloned in pLf0033 using restriction sites *kpn*I/*Eco*RI and HR2 using *Eco*RI/*Aat*II. The *pvcsp* alleles *pvcsp-vk210* (GenBank accession number P08677; Belem strain) and *pvcsp-vk247* (GenBank accession number M69059.1; Papua New Guinea strain) were amplified from existing plasmids PbG01-PvCSP-vk247 and PbG01-PvCSP-vk210 [27] using the primers P5/P6 and cloned into pL0033 containing the HR using restriction sites *Eco*RV/*Eco*RI, resulting in intermediate plasmids pLf0040 and pLf0041 (*pvcsp-vk210* and *pvcsp-vk247*; see **Figure S1**). An additional plasmid, AS301 (see Additional file 1) was used to clone the guide sgRNA (AS301-sgRNA2) specific for *pfcsp*. The sgRNA sequence was identified using the Protospacer software (alphaversion; https://sourceforge.net/projects/protospacerwb/files/Release/) and was amplified using the primers P7/P8. This sgRNA was selected based on the best-off targets hits score throughout the genome given by Protospacer and the total number of mismatches of the sgRNA with respect to the PAM site. A 20 bp guide sgRNA sequence, flanked on both sides by a 15 bp DNA sequence necessary for In Fusion cloning (HD Cloning Kit; Clontech), was annealed and used to replace the BtgZI adaptor as previously described [43]. The construct was then digested with *Bln*I and *Nru*I to evaluate the successful cloning of the sgRNA and later confirmed by Sanger sequencing using primers P9/P10. Finally, the AS301-sgRNA2 was digested with EcoR*V*/Apa*I* and cloned into the vectors pLf0040 and Plf0041 using the restriction sites *Stu*I/*Apa*I resulting in the final constructs pLf0042 and pLf0043 (see **Figure S1**).

Plasmids for transfection were isolated from 250 ml cultures of *Escherichia coli* XL10-Gold Ultracompetent Cells (Stratagene) by maxi-pep (using HiSpeed® Plasmid Maxi Kit (Qiagen®)) to generate 25-50 µg of DNA used per transfection. Transfections of *P. falciparum* NF54 parasites were performed using ring stage parasites obtained from cultures with a parasitaemia of 6-15% that were synchronized by 5% D-sorbitol treatment 2 days before transfection [44]. Infected RBC were pelleted by centrifugation (1150 *g*, 5 min) and 300 µl of the pelleted cells were transferred to a 0.2 cm cuvette and mixed with ~50 µg of each circular plasmid (Cas9 and sgRNA/Donor DNA constructs) in 100 µl cytomix [45]. Electroporation was performed with a single pulse (310 V and 950µF) in the Biorad Gene

Pulser Xcell electroporator (including CE- and PC module) and cells were immediately transferred in a 10 ml culture flask and cultures were maintained under standard conditions in the semi-automated culture system (see above). Selection of transformed parasites was performed by applying 'double' positive selection 24 h after transfection using the drugs WR99210 (2.6 nM) and BSD (5 µg/ml). For WR99210 100 µl of a stock solution (2.6 µM) was added to 100 ml complete culture medium resulting in a final concentration of 2.6 nM. To prepare the WR99210 stock-solution WR99210 was dissolved in DMSO (100mM). For BSD 50 µl of a stock solution (10mg/ml) was added to 100 ml complete culture medium resulting in a 5 µg/ml final concentration. Drug pressure in the cultures was maintained until thin blood-smears were parasite-positive (usually after 14 to 26 days). Positive selection will select for the parasites that were transfected successfully with both plasmids (Cas9 and sgRNA/Donor constructs). Subsequently, both drugs were removed from the cultures for 2-4 days, followed by applying negative selection by addition of 5-Fluorocytosine (5-FC; 130 µl of a stock solution (0.77 mM) in 100 ml complete medium with a final concentration of 1 µM; [46]) in order to eliminate parasites that retained the crRNA/Donor construct as episomal plasmid and enrich for the transfected parasites containing the donor DNA integrated into the genome. Negative drug pressure in the cultures was maintained until thin blood-smears were parasite-positive (usually after 7 days). After negative selection infected RBC (iRBC) were harvested from cultures with a parasitaemia of 4 to 10% for genotyping by diagnostic PCR and Southern blot analysis (see next sections). Subsequently, selected parasites were cloned by limiting dilution (see next sections).

Generation and selection of the PfΔcsp line

In order to create the *PfΔcsp* line, a new plasmid (pLf0070: that contain both the crRNA and the Cas9-expression cassette, was modified in order to introduce sgRNAs against *Pfcsp*. This plasmid, kindly obtained from Dr. Marcus Lee (Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK) is based on plasmid pDC2-cam-Cas9-U6.2-hdhfr [47] with a smaller U6 cassete (693bp) and with the Cas9 gene harmonized to *P. falciparum*. Two plasmids with two different sgRNAs (026 and 012) were generated. Briefly, pLf0070 was digested with *Bbs*I and sgRNA026 was cloned using the primers P11/P12 and sgRNA012 using the primers P13/P14. The primers (100 µM each primer) were phosphorylated with T4 polynucleotide kinase (10 Units per reaction) during 30 min at 37°C, followed by an annealing program of 5 min incubation at 94°C and a ramp down to 25°C at 5°C per min, and subsequently ligated into the vector using T4 ligase (5 units) resulting in plasmid pLf0071 and plasmid pLf0072 (see **Figure S1**). A second DNA donor plasmid was generated by replacing the *Pvcsp-vk210* gene of the pLf0040 construct (see above) by an mCherry expression cassette obtained from an intermediate plasmid pLf0055 (see **Figure S1**). In this cassette mCherry is under control of the promoter region of *gapdh* (GeneID PF3D7_1462800) and the 3'UTR of histidine-rich protein II(GeneID PF3D7_0831800). The complete mCherry expression cassette was removed from pLf0055 by digestion with the *EcoR*I/*Nru*I and cloned into pLf0040 digested with *EcoR*I/*EcoR*V resulting in the DNA donor vector pLf0083 (see **Figure S1**). This donor DNA construct has a drug selectable marker cassette containing a fusion of the positive selectable marker *hdhfr* and the negative selectable marker y*fcu* (*yeast cytosine deaminase/uridyl phosphoribosyl transferase).*

Transfections of *Pf* NF54 parasites was performed by spontaneous plasmid uptake from plasmid-loaded red blood cells cultured under static conditions [48]. Briefly, 300 µl of pelleted, uninfected RBC were transferred to a 0.2 cm cuvette and mixed with 50 µg of both sgRNA constructs (25 µg of pLf0071 and 25µg of pLf0072) and 50µg of the donor construct (pLf0083) suspended in 200 µl of cytomix. Electroparation was performed as described in the previous section. After electroporation of the uninfected RBC, iRBC containing *P. falciparum* NF54 parasites were added to a concentration of 0.1%. Selection of transformed parasites was performed when cultures reached a parasitaemia of 3% (after approx. 3 days) with 100 µl of WR992010 (2.6nM) during a period of 6 days. Subsequently the drug was removed and parasites were harvested at 0.8% of parasitaemia for mCherry fluorescence microscopy analysis to determine the ratio of wild type and mutant parasites present in the population. Parasites were collected from cultures that contained >80 mCherry-positive parasites (at a 4% to 10% parasitaemia) for genotyping by diagnostic PCR and Southern blot analysis and for cloning (see next section).

Cloning of transfected P. falciparum parasites

Based on the PCR confirmation of the integration, the transfected parasites were cloned by the method of limiting dilution as previously described [49] with minor modifications. Briefly, infected RBC from cultures with a 4% to 10% parasitaemia were diluted with uninfected RBC to 10^5 iRBC/100 µl in 2 ml culture medium (1% haematocrit and 20% serum). Serial dilutions were then performed with uninfected RBC in complete medium (1% haematocrit and 20% serum) and cultured in a total volume of 100 µl incubated in 96 well plates, resulting in 8 rows with the following numbers of iRBC per well: 100, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15. Plates were incubated in a Candle Jar at 37°C and culture medium was changed every other day. Every 5 days RBC were added resulting in an increase of the haematocrit from 1% to 5%. Between days 10-14 samples were collected for thick smear analysis from the rows with the highest numbers of iRBC/well; 50 µl medium was removed and from the remaining culture 5 µl was used directly for preparing thick smears. At day 21 thick smears were made from all rows. Clones were selected from dilutions/row with less than 30% of the wells parasite positive. These clones were transferred in 10 ml culture flasks at 5% haematocrit under standard culture conditions (see previous sections) in the semi-automated culture system for collection of parasites for further genotype and phenotype analyses (see next section).

Genotyping of the pf-pvcsp(vk210), pf-pvcsp(vk247) and pfΔcsp lines

For genotyping of the chimeric *pf-pvcsp* lines and the *pfΔcsp* line diagnostic PCR and Southern blot analysis of digested DNA were performed from material isolated from iRBC

obtained from 10ml cultures (parasitaemia 3 - 10%), pelleted by centrifugation (1150 *g*; 5 min.). RBC were then lysed with 5-10 ml of cold (4°C) erythrocyte lysis buffer (10x stock solution 1.5 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M Na_2EDTA; pH 7.4; [44]) and parasites were treated with RNAse and proteinase-K before DNA isolation by standard phenol-chloroform methods. Correct integration of the donor constructs was analysed by standard and long-range PCR (LR-PCR). In brief, for the chimeric *pf-pvcsp* lines integration of the *pvcsp* cassettes was confirmed by LR-PCR using the primers P15/P16 (and analysed by *EcoR*V digestion). The PCR-amplified product was cloned in a TopoTA vector for sequencing (see **Table S1** for details of the primers and **Figure S2** for sequence data). The LR-PCR fragments were amplified using KOD Hot start polymerase following standard conditions with an annealing temperature of 53.5°C for 15 sec and an elongation step of 68°C for 9 min. For the *Pf*Δ*csp* line, 5'-integration PCR was performed using the primers P15/P19 and to confirm the presence of the mCherry gene PCR was performed with the primers P20/P21. The PCR fragments were amplified using Go-taq® DNA polymerase (Promega) following standard conditions with an annealing temperature of 56°C for 20 sec and a elongation step of 72°C for 4 min. All other PCR settings were according to manufacturer's instructions.

Southern blot analysis for the chimeric *pf-pvcsp* lines was performed with genomic DNA digested with *Ava*II (4 hrs at 37°C) in order to confirm integration of the replacement of *pfcsp* by the *pvcsp* genes Fig. 1C). Digested DNA was hybridized with probes targeting the *Pfcsp* homology region 2 (HR2), amplified from NF54 genomic DNA by PCR using the primers P3/P4 and a second probe targeting ampicillin (Amp) gene, obtained by digestion of the intermediate plasmid pLf0040 with *Aat*II/*Pvu*I (550 bp). For Southern blot analysis of *Pf*Δ*csp*, genomic DNA was digested with *Ava*II and *Xho*I (4 hrs at 37°C) and digested DNA was hybridized with the same probes used with the *Pf-Pvcsp* lines (HR2 and Amp probes see Additional file 5 C).

Phenotype analysis of P. falciparum parasites: blood stages, gametocytes, oocysts and sporozoites

The growth rate of asexual blood stages of the *pf-pvcsp* and *pf*Δ*csp* lines was monitored in 10 ml cultures maintained in the semi-automated culture system under standard culture conditions (see above). Briefly, a 0.5% parasitaemia culture was established in complete culture medium at a haematocrit of 5%. Medium was changed twice daily and the culture maintained for a period of 5 days without refreshing RBC. For determination of the course of parasitaemia, triplicate samples of 100 µl were collected daily from all cultures and cells pelleted by centrifugation (9485 *g* ; 30 s) and stained with Giemsa. mCherry expression of in *Pf*Δ*csp* blood stages was analysed by standard fluorescence microscopy. In brief, 200 µl samples were collected from 10 ml cultures with a parasitaemia between 4 and 10% and stained with the DNA-specific dye Hoechst-33342 by adding 4 µl of a 500 µM stock-solution (final concentration 10 µM) for 20 min at 37°C. Subsequently, a 5 µl drop was placed on a microscopic slide (mounted under a cover slip) and fluorescence in live

iRBC analysed using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software and with the following exposure times: mCherry 0.6 sec; Hoechst-33342 0.136 s; bright field 0.62 s (1x gain).

Gametocyte production by the *pf-pvcsp* and *pf*Δ*csp* lines was analysed in gametocyte cultures, established as described in the previous sections. To activate gametocytes for exflagellation 20 µl samples of the *P. falciparum* stage V gametocyte cultures at day 14 were diluted 1:1 with FCS at room temperature. Gametes and exflagellation centres were examined and quantified 10-20 min after activation using a Bürker cell counter. For analysis of mosquito stages (oocysts and sporozoites) of the chimeric *pf-pvcsp* lines, *An. stephensi* were infected using the standard membrane feeding assay (SMFA) [50, 51]. Oocysts were analysed between day 8 and 14 for sporozoite production and the percentage of degenerated oocyst determined. Oocysts were qualified as degenerated based on the following criteria: no sporozoite formation visible and oocyst cytoplasm vacuolated. Salivary gland sporozoites were counted at day 14 and 21 post feeding. For counting sporozoites, salivary glands from 30-60 mosquitoes were dissected and homogenized using a grinder in 100 µl of RPMI pH 7.2 and sporozoites were analysed in a Bürker cell counter using phase-contrast microscopy.

Analysis of PvCSP expression in oocyst-derived sporozoites of the pf-pvcsp(vk210) and pf-pvcsp(vk247) lines

To analyse CSP expression in oocyst-derived sporozoites of the *pf-pvcsp* lines by immunofluorescence microscopy, midguts from 30-60 *An. stephensi* mosquitoes were collected in an eppendorf tube at day 10 after feeding in RPMI-1640 medium containing 3% BSA. Midguts were mechanically crushed using a grinder and centrifuged with low speed (62 *g*) for 3 min at 4°C. Subsequently, the supernatant containing oocyst-derived sporozoites was collected for fluorescence microscopy and samples (20 µl) were placed on a 8-well black cell-line diagnostic microscope slide (Thermo Scientific), dried for 10 min, and fixed with 4% paraformaldehyde for 30 min at room temperature. After fixation the slides were washed three times with 1X PBS and permeabilized with 20 µl of 0.5% triton in 1X PBS and then blocked with 10% of FCS in 1X PBS for 1h . Fixed cells were washed with 1X PBS and incubated with monoclonal antibodies against PvCSP(vk210) (mouse, anti-PvCSP-VK210 MAb (MR4); 1:200 dilution of 109µg/ml stock solution [27]) PvCSP-(vk247) (mouse, *anti*-PvCSP-VK247 MAb (MR4); 1:200 dilution of 125µg/ml [27]), PfCSP (mouse, anti-PfCSP (210 A) MAb(MR4); 1:200 dilution of 8µg/ml stock solution REF) and PfHSP70 (rabbit, anti-PfHSP70; 1:200 dilution of 100µg/ml stock solution StressMarqBiosciences) for 1h at room temperature. Subsequently, cells were rinsed 3 times with 1X PBS and incubated with the secondary antibodies Alexa FLuor®488/594-conjugated chicken anti-mouse and anti-rabbit (Invitrogen Detection technologies at 1:200). Finally, the cells were washed again three times with 1X PBS and stained with the DNA-specific dye Hoechst-33342 at

a final concentration of 10μM. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and a coverslip placed on the cells and sealed with nail polish. Stained cells were analysed for fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa 488: 0.7 sec; Alexa 594: 0.6 sec Hoechst 0.136 sec; bright field 0.62 sec (1x gain).

Phenotype analysis of Plasmodium berghei parasites: oocysts, sporozoites and sporozoite infectivity

Feeding of *An. stephensi* mosquitoes with *P. berghei* parasites, determination of oocyst production and sporozoite collection were performed as described [27]. Determination of parasite liver load by in vivo imaging and determination of the prepatent period in mice after intravenously injection of 1000 salivary gland sporozoites was performed as described [27].

Statistics

Data were analysed using GraphPad Prism software package 5.04 (GraphPad Software, Inc). Significance difference analyses between WT, *pf-pvcsp* and the *pfΔcsp* lines was performed using the unpaired Student's *t*-test,

Ethics approval

All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee for use under Project License 30/2889 and P9804B4F1.

Acknowledgements

C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). A.S. Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). Takashi Imai was, in part, supported by Uehara Memorial Foundation grant. The authors are grateful to Dr. Marcus Lee (Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK) for kindly providing us with a modified sgRNA-Cas9 expression plasmid, pDC2-cam-Cas9-U6.2-hdhfr (which we have termed pLf0070).

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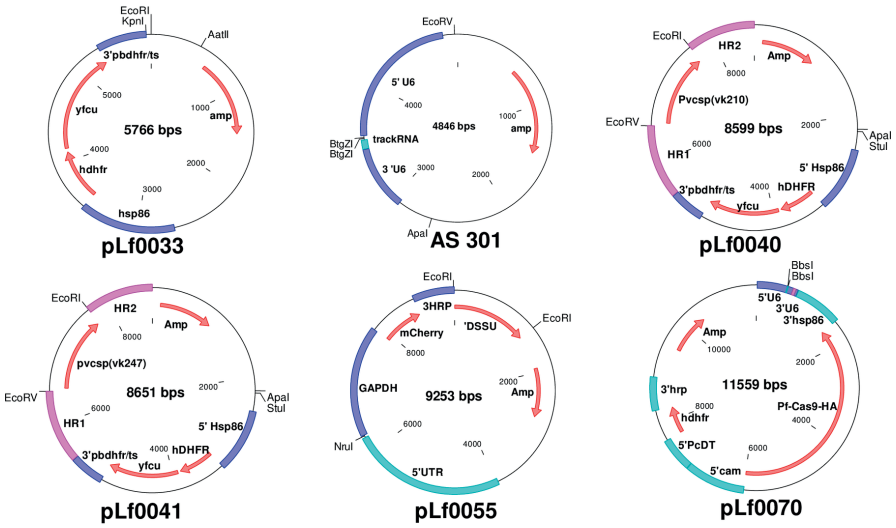
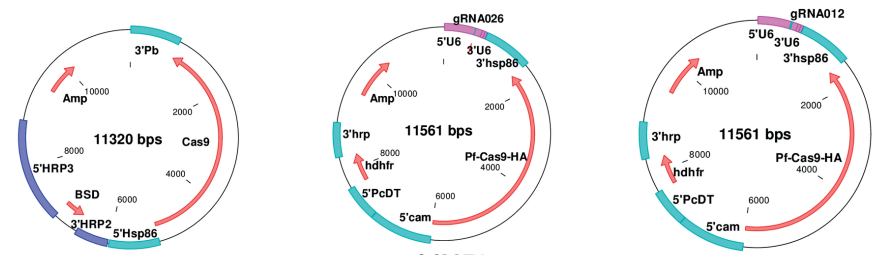
Supplementary Data

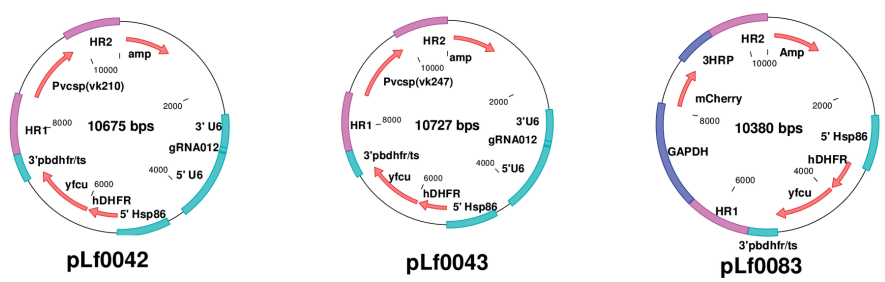
Intermediate plasmids



Cas9/sgRNA-Cas9 expression plasmids

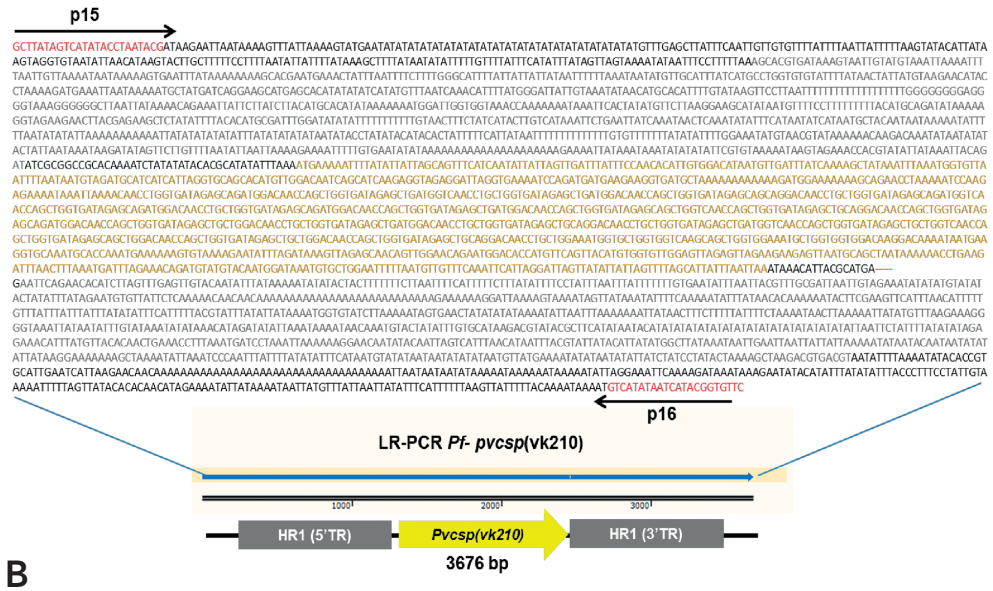


sgRNA-donor DNA/ donor DNA plasmids



Supplementary figure 1. Vector maps of the *P. vivax csp* genes introduced into *P. falciparum*. **A.** Vector maps of the different plasmids used to generate two chimeric *P. falciparum* parasite lines (*pf-pvcsp*) expressing *P. vivax* CSP(VK210) of CSP(VK247) and a *P. falciparum* line lacking expression of CSP (*Pf*Δ*csp*). See Methods section for description and details of the generation of these plasmids.

A



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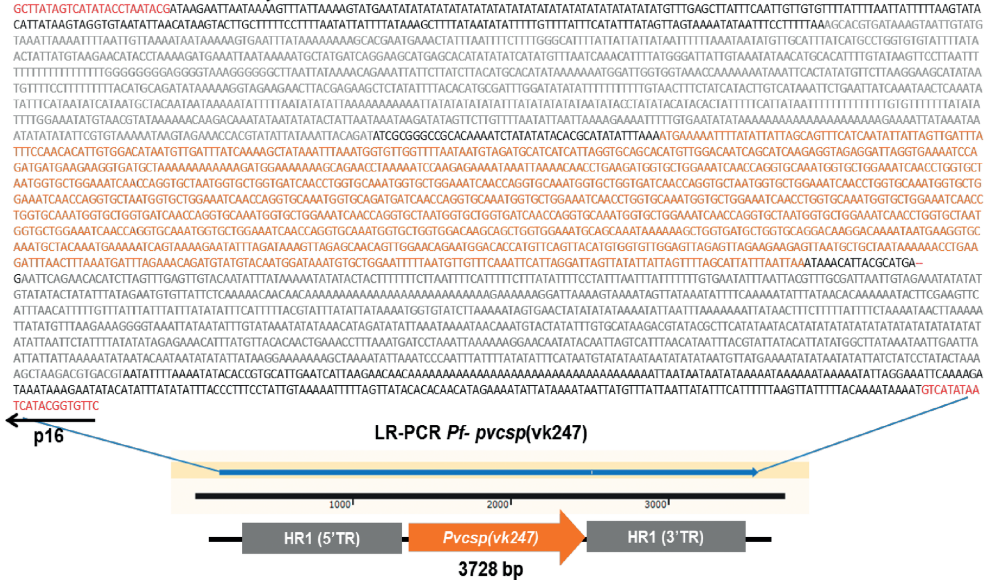
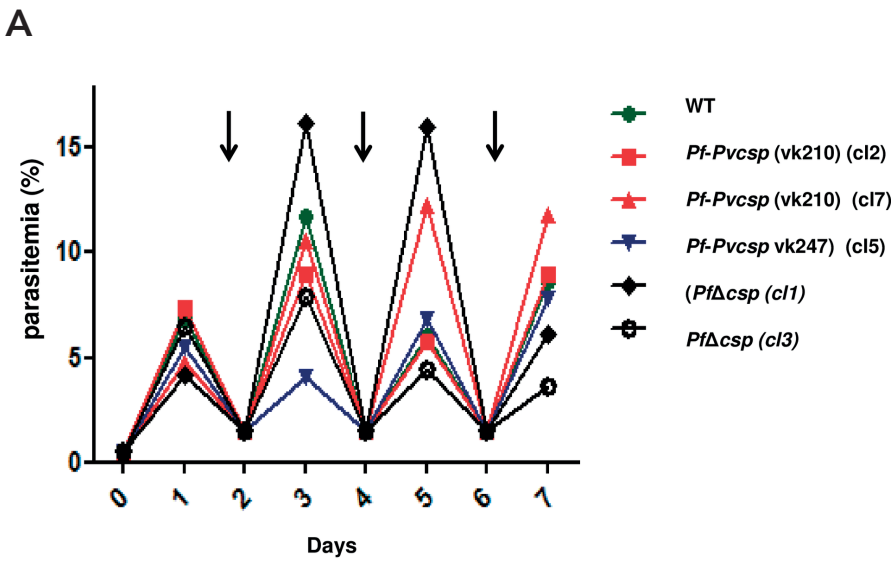
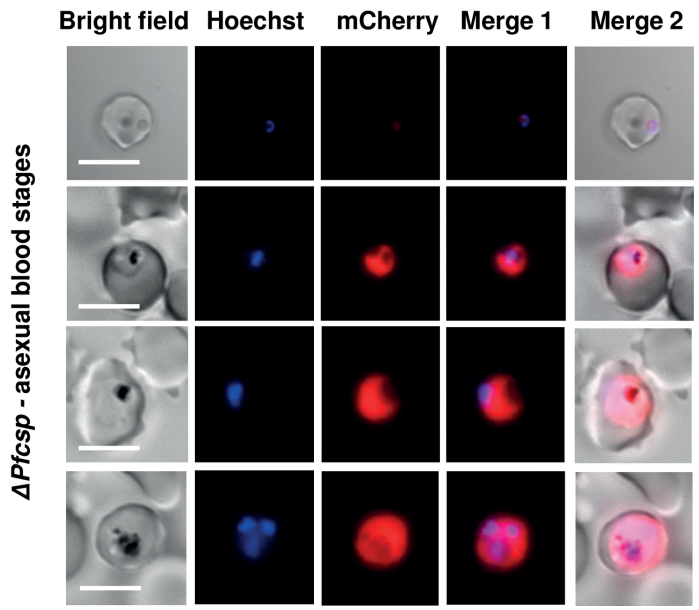
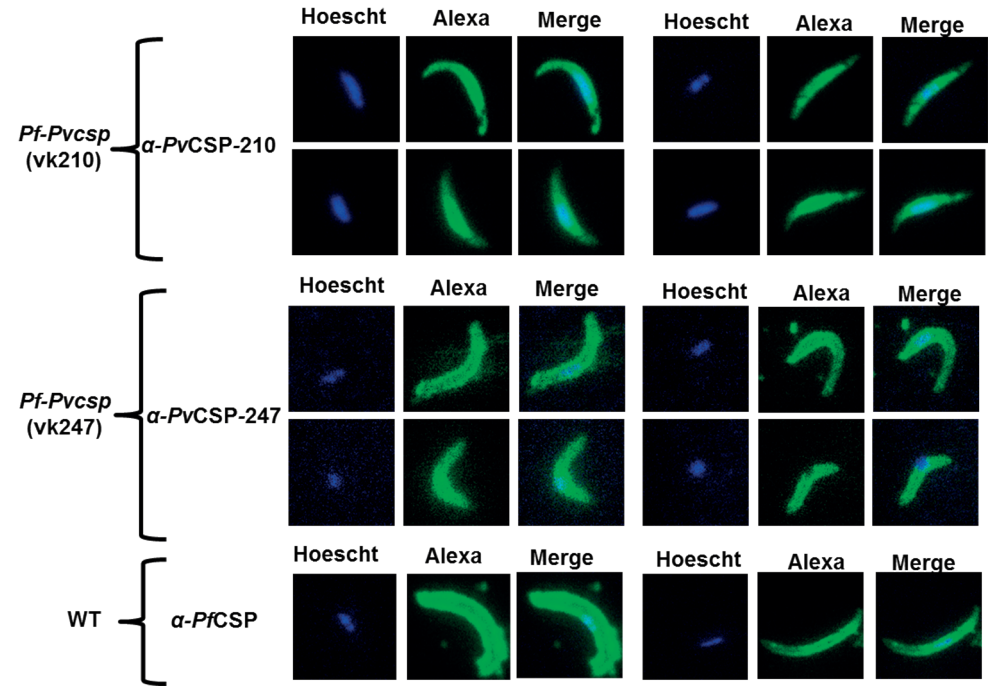


Supplementary figure 2. Sequence analysis of the *P. vivax csp* genes introduced into *P. falciparum*. Long-range PCR fragments (see Fig. 1) were cloned into pET TOPO TA (Invitrogen) and sequenced. See Additional file 7 for primer sequences used for sequencing the complete fragment. **A.** LR-PCR fragment of parasites of *pf-pvcsp*(vk210) **B.** LR-PCR fragment of parasites of *pf-pvcsp*(vk247)

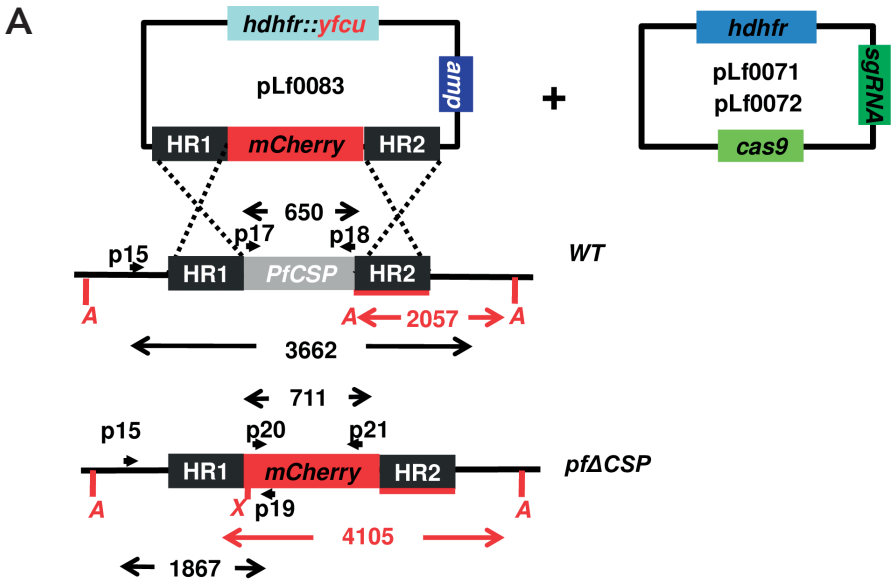




Supplementary figure 3. Growth of asexual blood stages and mCherry expression in blood stages. **A.** Growth of asexual blood stages of two chimeric *P. falciparum* parasite lines (*pf-pvcsp*(vk210) and *pf-pvcsp*(vk247), a *P. falciparum* line lacking expression of CSP (*Pf*Δ*csp*) and *P. falciparum* wild type (WT) parasites. Parasites of the different cloned lines were cultured in semi-automated culture system for a period of 7 days. Cultures were initiated with a parasitaemia of 0.5%. Arrows indicate the dilution of the cultures with fresh red blood cells to have a final parasitaemia of 1%. **B**. mCherry-expressing blood stages of *Pf*Δ*csp* parasites where the *csp* gene has been disrupted by insertion of an mCherry expression cassette (see Additional file 4 for details of the generation of *Pf*Δ*csp*). Scale bar, 7μm.

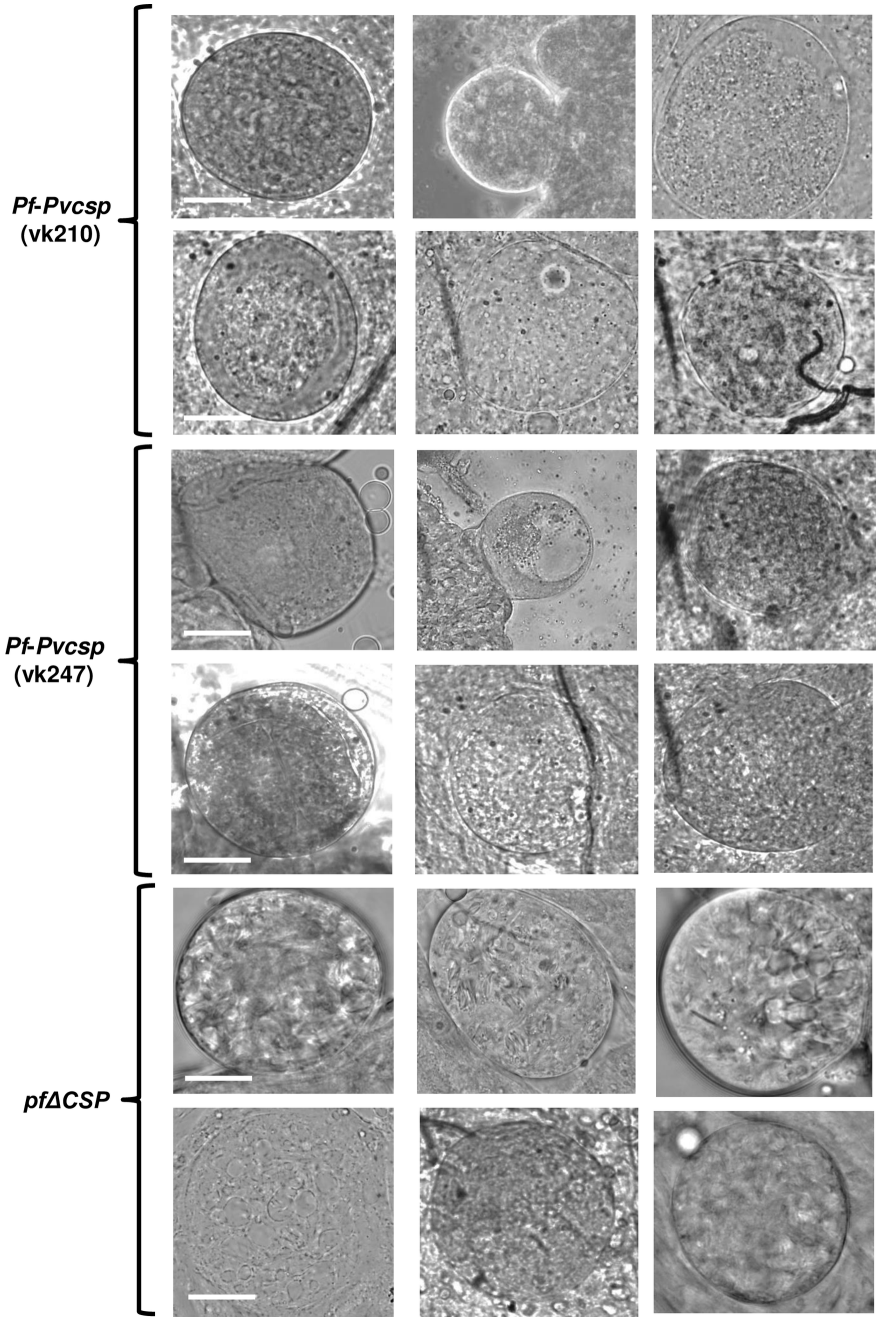


Supplementary figure 4. PvCSP(VK210) and PvCSP(VK247) expression in oocyst-derived sporozoites of two chimeric *P. falciparum* parasite lines *(pf-pvcsp)*. Immunofluorescence analyses of wild type *P. falciparum* (WT) sporozoites and oocyst-derived *pf-pvcsp* sporozoites. Fixed sporozoites were labelled with mouse anti-PvCSP-VK210 mAb, anti-PvCSP-VK247mAb and mouse anti-PfCSP antibodies. Secondary conjugated antibodies used: anti-IgG Alexa Fluor® 488 (green. Nuclei stained with the DNA-specific dye Hoechst-33342. All pictures were recorded with standardized exposure/ gain times; Alexa Fluor® 488 (green) 0.7 s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). Scale bar, 7μm.

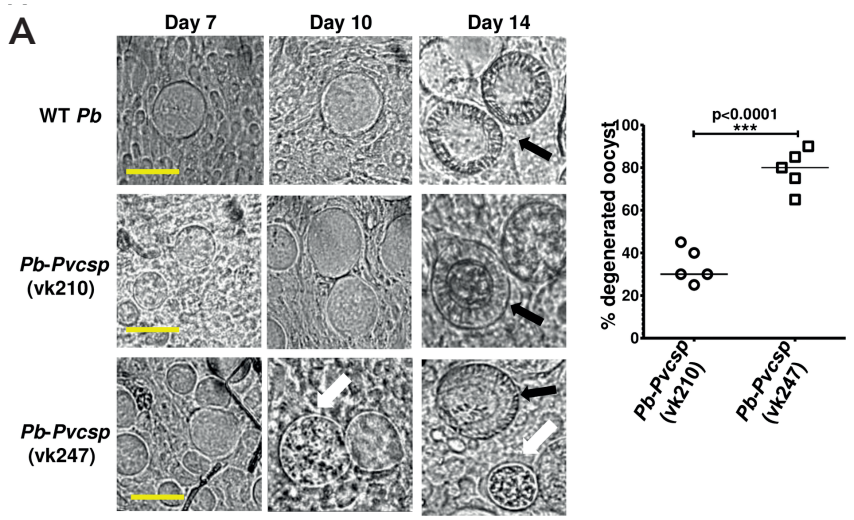


Supplementary figure 5. Generation and genotyping a *P. falciparum* mutant line lacking expression of CSP *(PfΔcsp)*. **A**. The *PfΔcsp* line was generated using CRISPR/Cas9 methodology. The *pfcsp* gene was replaced by insertion of a mCherry expression cassette (mCherry under control of the of the constitutive *gapdh* promoter) using donor-DNA plasmids pLf0086. A schematic representation of the *pfcsp* locus before and after insertion of the construct showing the location of the restriction sites (*A: Ava*II), sizes (in bp) of restriction fragments (red for Southern blot analysis), location of primers (p), PCR amplicons and sizes of the fragments (in black) used to analyse correct disruption of the *pfcsp* and insertion of the mCherry cassette (**B, C**). HR1, HR2: *pfcsp* homology (targeting) regions. The figure is not shown to scale. Primer sequences can be found in Additional file 7. **B**. Diagnostic

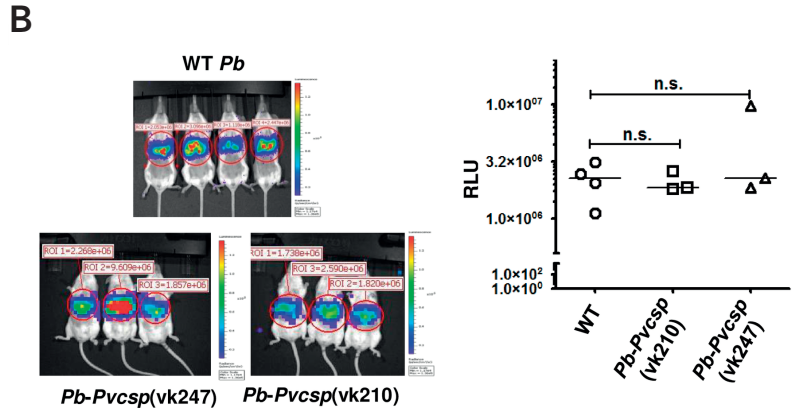
PCR confirming correct integration of the mCherry cassette into the PfCSP locus. 5' integration PCR (lane 2; primers p15/p19); *pfcsp* open reading frame (lane 3; primers p17/p18); *P. falciparum sequestrin* as a control gene (lane 1; primers p22/p23); mCherry gene (lane 4; primers P20/P21) of cloned parasites of *PfΔcsp* (cl3) and WT. **C.** Southern blot analysis of *Ava*II/*Xho*I restricted DNA of WT and *PfΔcsp* parasites confirms the specific integration of the mCherry cassette into the *pfcsp* gene locus. DNA was hybridized with a probe targeting the homology region 2 (upper panels; HR2; primers p3/p4; see **A**) of *pfcsp*. The hybridization pattern observed with the HR2 probe identified the expected different-sized DNA fragments in WT and *pf-pvcsp* parasites (2057 bp and 4105 bp). In addition to show absence of donor-DNA plasmid and presence of single cross-over events DNA was hybridized with a probe for the ampicillin gene (lower panels; intermediate donor-DNA plasmid pLf0040 digested with *Aat*II and *Pvu*I).



Supplementary figure 6. Degenerated oocysts of two chimeric *P. falciparum* parasite lines *(pf-pvcsp)* and a PfCSP knockout line *(PfΔcsp)*. Light microscope pictures of degenerated oocysts at day 10 after feeding gametocytes to *Anopheles stephensi* mosquitoes. These oocysts are classified as degenerate based on the absence of sporozoite formation and vacuolated cytoplasm. See Figure 2 for *pf-pvcsp* and wild type *P. falciparum* (WT) oocyst in which sporozoite formation occurred. No sporozoite formation was observed in *PfΔcsp* oocysts. Scale bar, 20µm.



Parasite line	Oocyst no. (mean ± D)	Sporozoite no. (mean ± SD)	Significant different from WT (Student t-test)
WT Pb	116 ± 22	22,800 ± 3453	NA
***Pb-Pvcsp*(vk210)**	112 ± 37	18,700 ± 3755	p = 0.11 (NS)
***Pb-Pvcsp*(vk247)**	101 ± 28	4,200 ± 1549	p < 0.0001 (****)



Supplementary figure 7. Chimeric rodent parasite lines *pb-pvcsp(*vk210) and *pb-pvcsp(*vk247) are able to produce salivary gland sporozoites that are infectious to mice. **A**. Upper panel, left: Light microscope pictures of wild type *P. berghei* (WT) and *pb-pvcsp* oocyst at days 7, 10 and 14 after feeding of *An. stephensi* mosquitoes. Black arrows indicate oocyst with sporozoite formation and white arrows indicate degenerated, vacuolated oocyst without sporozoite formation (scale bar, 10 µm). Upper panel, right, Percentage of degenerated oocyst in *An. stephensi* mosquitoes (n=5) at day 14 after feeding (***p=<0,0001; unpaired T-test). Lower panel: Oocyst and sporozoite production of wild type *P. berghei* (WT) and *pb-pvcsp* parasites. **B.** *In vivo* imaging of parasite liver loads at 44 h after injection of mice with salivary gland sporozoites of wild type *P. berghei* (WT) and *pb-pvcsp* parasites. Left panel: luminescence signals in the different groups of mice. Right panel: quantification of the bioluminescence signals in the different groups of mice measured as Relative Luminescence Units (RLU).

Supplementary Table 1. List of primers used in this study

Primer ID	Gene ID	Sequence	Enzymes	Product (bp)	Description
P1	PF3D7_0304600	ATAGGTACCAGCACGTGATAAAGTAATTG	*Kpn*I		Forward HR 1 *pfcs*
P2	PF3D7_0304600	ACGGAATTCCGGCGGATATCTGTAATTTATAATATACGTGG	*EcoR*I/*EcoRV*	1007	Reverse HR 1 pfcs
P3	PF3D7_0304600	CATAAGAATTCAGAACACATCTTAGTTTGAG	*EcoR*I		Forward HR 2 *pfcs*
P4	PF3D7_0304600	AATCGACGTCTTAGCTTTTAGTATAGGATAG	*Aat*II	927	Forward HR 2 *pfcs*
P5		GGGGATATCGCGGCCGCACAAAATCTATATATACACGCATATATTTAAAATG	*EcoRV*/*Not*I	1188	Forward ORF pvcsp-vk210/247
P6		AGAATGAATTCTCATGCGTAATGTTTATTTAATTAAATAATGC	*EcoR*I		Reverse ORF pvcsp-vk210/247
P7	PF3D7_0304600	TAAGTATATAATATTTAAGGCCTCAACAAATAAAAGTTTTAGAGCTAGAA			Forward gRNA012
P8	PF3D7_0304600	TTCTAGCTCTAAAACTTTTATTTGTTGAGGCCTTAAATATTATATACTTA			Reverse gRNA012
P9		CATTTGGATTTCTACACATCTTG			Sequencing gRNA012
P10		TAGGAAATAATAAAAAAGCACC			Sequencing gRNA012
P11	PF3D7_0304600	TATTATGGAAGTTCGTCAAACACA	*Bbs*I		Forward gRNA026
P12	PF3D7_0304600	AAACTGTGTTTGACGAACTTCCAT	*Bbs*I		Reverse gRNA026
P13	PF3D7_0304600	TATTTAAGGCCTCAACAAATAAAA			Forward gRNA012 *Pf csp ko*
P14	PF3D7_0304600	AAACTTTTATTTGTTGAGGCCTTA			Reverse gRNA012 *Pf csp ko*
P15	PF3D7_0304600	GCTTATAGTCATATACCTAATACG		3676,3728 (*Pf-Pvcsp*),	Forward long range PCR
P16	PF3D7_0304600	GAACACCGTATGATTATATGAC		3662 (WT)	Reverse long range PCR
P17		GATGGAAATAACGAAGACAACGAG		650	Forward *Pf csp* orf
P18		CATCTACATTTCGGTTTGGGTCA			Reverse *Pf csp* orf
P19		CCAATTATTGCTGATTTATACAAATG		With P15 1867	Reverse 5'integration
P20		AAAGGTACCTAAAAGAAATATGAGAAC		711	Forward mCherry
P21		AAAAAGCTTTTTCGCCACAGGAGAAAC			Reverse mCherry
P22	PF3D7_0405300	AACGCTAGCTTCCGGATCGCTGTCTTTAC			Forward Sequestrin
P23	PF3D7_0405300	AGCCGCGGCATGGAGAAGGGTTCTATTTTATCG		5400	Reverse Sequestrin
P24		CGCGTAATACGACTCACTATAGGGC			LR-PCR sequencing
P25		CAGTGTGATGGATATCTGCAG			LR-PCR sequencing
P26		CATGCAGATATAAAAAGGTAGAAG			LR-PCR sequencing
P27		AGCAGAACCTAAAAATCCAAG			LR-PCR sequencing
P28		CATTTCCACCAGCTGCTTG			LR-PCR sequencing
P29		CCGGGGTACCAGTTGTGTAACATAAATGTTTCTC			LR-PCR sequencing
P30		CATGTGGTGTTGGAGTTAGAG			LR-PCR sequencing
P31		TTTATGCTTCCGGCTCGTATG			LR-PCR sequencing
P32		ggggatatcgcggccgcACAAAATCTATATATACACGCATATATTTAAAATG			LR-PCR sequencing

CHAPTER 7

Conclusions and Discussion

Conclusions and discussion

In this thesis we describe a set of studies that employed novel CRISPR/Cas9 methodologies to improve *Plasmodium falciparum* genetic modification. This was done in order to create novel transgenic parasites, which can be used to better interrogate host-pathogen interactions or used in anti-malarial drug and vaccine research. We first focused on improving *P. falciparum* CRISPR/Cas9 gene editing technology and on identifying a suitable 'neutral' locus to introduce transgenes into the parasite genome. Using this improved CRISPR/Cas9 methodology, transgenic *P. falciparum* parasites were created that express either fluorescent-luminescent reporter proteins or express a major vaccine candidate from the other major human malaria parasite, *P. vivax*.

Improved CRISPR/Cas9 genetic modification of *P. falciparum* (Chapter 3)

The RNA-guided CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system has transformed genome editing in a wide variety of organisms [1]. This powerful genome editing technique has also been applied to *P. falciparum* and provides an efficient method for manipulating the parasite genome, including site-directed mutagenesis, gene disruption and the introduction of transgenes [2, 3].

In **Chapter 3** we describe further improvements of the CRISPR/Cas9 transfection constructs and selection protocol in order to more rapidly modify the *P. falciparum* genome and to introduce transgenes into the parasite genome without the inclusion of drug-selectable marker genes. This method was used to stably integrate three different GFP-expression cassettes into the *P. falciparum* genome, where GFP is under the control of promoters of three different *Plasmodium* genes. The selection procedure to obtain genetically modified *P. falciparum* parasites before the use of CRISPR/Cas9 methods required 1-3 months of continuous culture to select for parasites where episomal plasmids became integrated into the parasite's genome, either by single or by double cross-over recombination [4]. Moreover, the process of removing introduced drug-selectable markers from genetically modified parasites, which involved multiple steps of cloning, can typically take 5 months or more to complete [5]. With the methods described in **Chapter 3** we describe a protocol where cloned, drug-selectable marker-free parasite lines that stably express reporter proteins can be obtained within a period of 10-12 weeks. The CRISPR/Cas9 *Plasmodium* transfection method requires the introduction of two plasmids into the parasite by electroporation. In one of the plasmids, the sgRNA/donor plasmid, we have placed the selectable-marker cassette outside the donor-DNA cassette, which results in the introduction of a transgene into the parasite genome without the introduction of a drug-selectable marker. Hence the locus can be modified without the inclusion of a drug-selectable marker cassette. These constructs can easily be modified to also perform other genetic manipulations, notably gene-disruptions and gene-mutations, which can

be used to interrogate gene function and importance. A consequence of the absence of a drug-selectable marker in the genome is that these 'marker-free' parasites can be further modified more rapidly and easily to create parasites that have multiple genetic modifications, for example, 'double' modified parasites that contain both a reporter gene as well as a disrupted or mutated gene. Indeed, such rodent malaria mutants have been used extensively to analyse the phenotypic consequences of a gene disruption/mutation, *in vitro* and *in vivo*, using a variety of imaging technologies.

The GFP-expression cassettes were introduced into the *p230p* gene locus of the *P. falciparum* genome. The *p230p* locus was selected as we predicted it to be a 'neutral' locus based on rodent *Plasmodium* studies, i.e. the loss of this *P. falciparum* gene would not affect parasite development. However, disruption of the *p230p* gene unexpectedly resulted in *P. falciparum* parasites that could not infect and develop in mosquitoes. We describe in **Chapter 4** the phenotype of the '*p230p* gene-deletion' mutants in mosquitoes and the potential role of the P230p protein in mosquito development. In *P. falciparum* the *p47* gene locus has been most frequently used to introduce transgenes into the *P. falciparum* genome [6, 7]. We initially had selected the *p230p* gene locus to introduce transgenes as an alternative to the *p47* gene, since the P47 protein has been shown to be important for limiting defence responses against the parasite in mosquitoes [8, 9]. Consequently, *P. falciparum* parasites lacking P47 expression have a reduced capacity to transmit through some species of *Anopheles* mosquitoes, as they are less capable of escaping the mosquito immune response. In two rodent *Plasmodium* species the male-specific P230p protein appears to be dispensable throughout the parasite's complete life cycle [10-12]. *P. berghei* and *P. yoelii* mutants lacking expression of P230p can develop in the vertebrate host and in the mosquito vector without a discernible phenotype and *p230p* knock-out parasites manifest a wild type parasite phenotype. As rodent *Plasmodium* P230p is not essential, the *p230p* gene is a frequently used locus to introduce additional transgenes into rodent malaria parasite genomes [11]. In contrast, we found that *P. falciparum p230p* cannot be used as a 'neutral locus', in particular for the generation of genetically modified parasites that need to be examined in mosquitoes or in liver stages (see also **Chapter 4**).

Future studies

We generated a set of CRISPR/Cas9 constructs that can be modified and used in future studies to remove or introduce transgenes into the *P. falciparum* genome. For example, these can be used to i) generate reporter lines that express additional (fluorescent/bioluminescent) reporter proteins; ii) to analyse the genetic regulatory elements that control *Plasmodium* gene expression; iii) change the homology regions of the sgRNA/donor DNA construct to permit introduction of transgenes into different *P. falciparum* genetic loci; or iv) to introduce other transgene (e.g. reporter) expression cassettes under the control of different regulatory promoter and transcription terminator (5'- and 3'-UTR) regions.

In this study we generated three different reporter parasite lines, principally to identify strong constitutive promoters that can be used to drive reporter gene expression. We found that the *cam* promoter resulted in relatively weak GFP-expression compared to *gapdh* and *hsp70* in blood stage parasites, which is in agreement with *Plasmodium* RNAseq gene-expression data. The *Plasmodium hsp70* promoter has been used frequently to drive transgene expression in rodent reporter parasites. The *gapdh* promoter has not been extensively examined in either rodent or human parasites. GAPDH is an enzyme involved in glycolysis, the main pathway for ATP production in *Plasmodium* [13], and therefore this protein is likely to be expressed throughout the complete life cycle. Indeed, proteome analyses of oocysts and sporozoites provide evidence for high abundance of GAPDH in these stages (PlasmoDB; www.plasmodb.org). Therefore, we believe that both *hsp70* and *gapdh* promoters may be used to drive transgene expression throughout the complete parasite life cycle.

Further improvements could be made to the CRISPR/Cas9 methodology. In particular the replacement of the 'two plasmid' based transfection system, which results in reduced transfection efficacy and requires double drug selection, with a more efficient and rapid 'single plasmid' transfection method [14].

This study revealed that *230p* gene locus, unexpectedly, was not suitable for creation of transgenic parasite lines that could transmit through mosquitoes. Therefore, identification of additional 'neutral' genetic loci in *P. falciparum* is required to expand the possibilities for creating transgenic reporter lines that can be used at multiple points of the parasite life-cycle. This is even more pressing, since the most frequently used locus for introduction of transgenes is the *p47* gene, whose removal has been shown to compromise *P. falciparum* development in different strains of *Anopheles* mosquitoes.

The phenotype of *P. falciparum* mutants lacking P230p expression (Chapter 4)

The GFP-expression cassettes in the reporter *P. falciparum* lines described in **Chapter 3** were introduced into the genome in the *p230p* gene locus. As we mentioned above, disruption of the *p230p* gene resulted in parasites that could not infect and develop in mosquitoes. In **Chapter 4** we describe studies to analyse the phenotype of the *p230p* 'gene-deletion' mutants in mosquito stages and to identify its potential role in parasite fertilization.

The P230p protein belongs to the s48/45 domain 6-cysteine (6-cys) family of *Plasmodium* proteins, a small family with 14 members that show stage-specific expression throughout the parasite life cycle, with most members localizing to the surface of parasites [15]. Most members have critical roles in parasite development, either in the vertebrate host or in the mosquito vector, and several members are leading targets for malaria vaccines. These include vaccine antigens that target parasites in the mosquito, the so-called transmission blocking vaccine antigens, i.e. P48/45 and P230 [16-18]. In *P. falciparum* these proteins

are expressed in both male and female gametocytes/gametes [19-21]. In two rodent *Plasmodium* species the paralog of P230, the male-specific P230p protein, appears to be dispensable throughout the parasite's complete life cycle [10-12].

We found that *P. falciparum* mutants lacking P230p expression (*PfΔp230p*) produced wild type numbers of male and female gametocytes that retained expression of P48/45 and P230. Upon activation, male *PfΔp230p* gametocytes undergo exflagellation and form male gametes. However, these male gametes are unable to attach to red blood cells, resulting in the absence of characteristic exflagellation centres *in vitro*. In the absence of P230p, zygote formation as well as oocyst and sporozoite development were strongly reduced in mosquitoes. These observations demonstrate that P230p, like P230 and P48/45, has a vital role in *P. falciparum* male fertility and zygote formation and identifies P230p as one of a limited number of gamete-specific proteins critical for *P. falciparum* transmission.

The function of P230p in male gamete fertility is in agreement with male-specific expression of *Pf*P230p and concomitant absence in female gametocytes/gametes [19-24]. The important role that *P. falciparum* P230p plays in mosquito transmission does not match the redundant function of P230p in the rodent parasites *P. berghei* and *P. yoelii*. Rodent parasites also express P230p specifically in male gametocytes [25], but mutants lacking P230p have no discernible defect and exhibit normal gametocyte/gamete formation. Activated male gametocytes are fully able to form exflagellation centres and mosquito transmission is similar to WT parasites [10, 11].

In **Chapter 4** we also provide evidence that *P. berghei* P230 is not compensating for the loss of its paralogue P230p, since activated male gametocytes of *P. berghei* mutants lacking expression of both P230 and P230p can still bind to RBCs and form exflagellation centres. These observations demonstrate a critical difference in the function P230p performs in rodent and human malaria parasites. For a few other 6-Cys proteins evidence has been found for functional differences between the orthologs of rodent and human parasites. For example, the female-specific P47 protein is vital for the fertility of *P. berghei* female gametes, while this protein does not appear to be crucial for *P. falciparum* female gamete fertility [10, 26]. Analysis of *P. berghei* mutants lacking P45/48 and P230 demonstrates that these proteins are male-specific fertility factors [10, 27]. In contrast, *P. falciparum* P48/45 and P230 are expressed in both males and female gametes [19-21], which may suggest a role for these proteins in both male and female gamete fertility.

The lack of RBC binding of *PfΔp230p* male gametes is similar to the phenotype of *P. falciparum* mutants lacking expression of its paralog P230 [28]. These observations suggest that both proteins have a similar, but not interchangeable, function in RBC binding. We provide evidence that *PfΔp230p* male and female gametes retain expression of both P230 and P48/45, indicating that the lack of RBC binding is not due to the absence of expression of P230 and/or P48/45. These observations would suggest that RBC binding of male gametes is not due to direct interactions of P230 to RBC receptors as was also demonstrated in studies using male gametes lacking expression of P230 [28].

Future studies

P230 and P48 form complexes with several other proteins at the surface of female gametes and zygotes [29-31]. Given that P48/45, P230 and P230p are expressed in male gametes, it is conceivable that comparable complexes, including additional proteins, are also formed at the surface of male gametes. The absence of either P230 or P230p may affect the correct formation of such protein complexes at the gamete surface, which may in turn lead to the same loss of the RBC-binding phenotype observed in mutants that lack either paralog. Future studies are needed to unravel in more detail the molecular interactions between male gametes and RBC and the *Plasmodium* ligand(s) that bind to the putative protein receptors, sialic acid and/or glycophorin on the RBC surface [32].

Since both P230 and Pf48/45 are present on the surface of *P. falciparum* gametes and have a critical role in fertilization they are actively being pursued as malaria vaccine candidates. Immunization with these antigens provoke antibody-based responses in the host that could limit parasite transmission in mosquitoes, so called transmission blocking vaccination [33]. Therefore, our observations of the vital role of P230p in *P. falciparum* fertilization and zygote formation, like P230 and P48/45, would indicate that studies should be performed to investigate its potential as a transmission blocking vaccine candidate antigen; for example, to examine whether immunization with P230p could also generate antibody responses that could limit parasite development in mosquitoes.

A *P. falciparum* NF54 reporter line expressing mCherry-luciferase in gametocytes, sporozoites and liver stages (Chapter 5)

In **Chapter 3** we described studies to generate and analyse different *P. falciparum* transgenic lines expressing GFP from different promoters. This was performed in order to create highly fluorescent reporter parasites that express GFP throughout the complete life cycle. However, given that the insertion of a transgene into the *P. falciparum p230p* locus resulted in parasites that could not infect mosquitoes (**Chapter 3 and 4**), we reverted to using the standard 'neutral' *p47* gene locus for introduction of a novel reporter expression cassette. In **Chapter 5** we describe the creation and evaluation of a reporter line that expresses a fusion of mCherry and luciferase, driven by the *etramp10.3* gene promoter. We selected the *etramp10.3* promoter as this gene has structural similarity to the rodent *Plasmodium uis4* gene and *etramp10.3* and *uis4* have the same syntenic genomic location. In multiple rodent malaria transgenic lines the *uis4* promoter has been used to drive expression of different transgenes, specifically in sporozoites and liver stages, such as genes encoding mCherry, ovalbumin or human malaria proteins [34-40]. We chose to generate an mCherry-expressing *P. falciparum* line in order to visualise interactions of *Plasmodium* sporozoites with host-cells (e.g. immune cells or hepatocytes) that are often labelled with green fluorescent proteins. Moreover, we fused the mCherry gene to the gene encoding firefly luciferase as luciferase expression can be used to quantify parasite numbers (e.g.

sporozoites and liver-stages) using simple and sensitive luminescence assays [41-43]. Using the CRISPR/Cas9 methodology, described in **Chapter 3**, we inserted the *mCherry-luc@etramp10.3* expression cassette into the *p47* gene locus. We demonstrated that this transgenic parasite line (*mCherry-luc@etramp10.3*) expresses mCherry in gametocytes, sporozoites and liver-stage parasites. While we did not detect mCherry above background levels in asexual blood-stage parasites, we were able to measure luciferase expression in asexual blood-stages as well as in gametocytes, sporozoites and liver-stages. The highest levels of reporter expression were detected in stage III-V gametocytes and in sporozoites.

The high activity of the *etramp10.3* promoter in gametocytes is in agreement with the high levels of *etramp10.3* transcripts and ETRAMP10.3 protein levels previously reported in gametocytes by genome-wide analyses of gene expression. The peak *etramp10.3* transcript abundance was observed in stage III gametocytes [44], and ETRAMP10.3 is detected in proteomic analyses of (male and female) gametocytes [20, 21] and is more abundant in gametocytes compared to asexual blood-stages. The expression of ETRAMP10.3 in asexual blood-stages has been reported in proteomic analyses [45, 46] and has been confirmed by immunofluorescence analysis using anti-ETRAMP10.3 antibodies [47]. Moreover, unsuccessful attempts to delete the gene *etramp10.3* indicates that it is essential during asexual blood-stage development [47]. A vital role for ETRAMP10.3 during blood-stage development is in contrast to the rodent *Plasmodium* UIS4 protein, which is dispensable for blood-stage development [48, 49]. In previous studies using rodent parasites, where the *usi4* gene has been replaced with the *etramp10.3* gene, ETRAMP10.3 is unable to complement the essential function of UIS4 in *P. yoelii* liver-stages [47]. These observations indicate that UIS4 and ETRAMP10.4 may have different or only partially overlapping roles in rodent and human malaria parasites. The expression of mCherry and luciferase in gametocytes and sporozoites makes this transgenic parasite line suitable to use in *in vitro* assays to examine the effect of drugs/inhibitors and vaccine-induced immune responses on gametocyte and sporozoite development, as well as to analyse sporozoite biology in mosquitoes, skin and hepatocytes.

Future studies

The development and expansion of reporter lines in *P. falciparum* will increase the range of analyses that could be performed, as has been the case with reporter lines of the rodent parasites *P. berghei* and *P. yoelii*. Studies with *P. falciparum* transgenic reporter parasites, possibly using protocols established with rodent malaria reporter lines, may help to better interrogate *P. falciparum* gene function at different points of development, and will enhance screening of compounds or immune sera in miniaturized, high throughput and rapid assays that inhibit the parasite at different points of development. For example, mCherry-luciferase expressing parasites, such as described in this study, could be used to better understand the interactions of sporozoites and host cells (i.e. in the skin) as well as to examine the action of drugs or vaccines that target gametocyte or liver stage parasites.

Additional reporter lines are required for *P. falciparum*, in particular those that show high reporter expression throughout liver stage development (i.e. stronger than either *GFP-luc@ef1α* or *mCherry-luc@etramp10.3*), both for sensitive drug tests as well as for the possibility of flow sorting of infected hepatocytes. We are currently creating and testing next-generation *P. falciparum* reporter lines, using data from multi-stage RNAseq transcription analyses to identify suitable strong and constitutive promoters.

Chimeric *Plasmodium falciparum* parasites expressing *Plasmodium vivax* circumsporozoite protein fail to produce salivary gland sporozoites (Chapter 6)

Rodent malaria parasites where the gene encoding circumsporozoite protein (CSP) has been replaced with *csp* genes from the human malaria parasites, *P. falciparum* (*Pf*) or *P. vivax* (*Pv*), are being used as pre-clinical tools to evaluate vaccines targeting CSP *in vivo*. These chimeric rodent parasites produce sporozoites in *A. stephensi* mosquitoes that are capable of infecting rodent and human hepatocytes. The availability of chimeric *P. falciparum* parasites where the *Pfcsp* gene has been replaced by *pvcsp* would open up possibilities for testing *P. vivax* CSP vaccines in small scale clinical trials using controlled human malaria infection (CHMI) studies. Testing the next generation of *P. falciparum* vaccines and vaccine formulations is greatly aided by the ability to vaccinate individuals and then examine vaccine efficacy by infecting immunized individuals with WT malaria parasites in controlled human malaria infections (CHMI)[50-53] Although recently CHMI has also been developed for *P. vivax* [54] and has been applied to assess pre-erythrocytic vaccine candidates [22, 23], *P. vivax* CHMI to screen different *P. vivax* vaccines is extremely limited, due in large part to the lack of methods to continuously propagate *P. vivax* blood stages in culture and to produce gametocytes *in vitro* that can be used to infect mosquitoes and produce sporozoites for challenge infections [54]. *P. vivax* CHMI is largely dependent on sporozoites which have been obtained from mosquitoes fed on infected patients [54]. We reasoned that the availability of chimeric *P. falciparum* parasites that express *P. vivax* antigens would open up possibilities to analyse protective immune responses induced by *P. vivax* antigen-based vaccines followed by CHMI with chimeric *Pf-Pv* parasites, bypassing the need for *P. vivax* sporozoite production and, equally importantly, eliminating the measures required to ensure that *P. vivax* dormant liver stages, hypnozoites, have been removed. In **Chapter 6**, we describe proof-of-concept studies where we generated two chimeric *P. falciparum* parasites using CRISPR/Cas9 gene editing methodologies, in which the *pfcsp* gene was replaced by one of the two major *pvcsp* alleles, either VK210 or VK247 [55].

We found that the two chimeric Pf-PvCSP lines exhibit normal asexual and sexual blood stage development *in vitro* and produce sporozoite-containing oocysts in *A. stephensi* mosquitoes. Expression of the corresponding *Pv*CSP was confirmed in oocyst-derived Pf-PvCSP sporozoites. However, most oocysts degenerate before sporozoite formation and

sporozoites were not found in either the mosquito haemocoel or in salivary glands. Unlike the chimeric Pf-PvCSP parasites, oocysts of *P. falciparum* parasites lacking CSP-expression do not produce sporozoites. These observations indicate that the *Pv*CSP proteins can be used to initiate sporozoite formation in *P. falciparum* oocysts but are unable to fully complement the function of *Pf*CSP in oocyst maturation and sporozoite development. It seems unlikely that the failure of *Pv*CSP to functionally complement *Pf*CSP is due to incorrect expression of the *Pv*CSP proteins in the Pf-PvCSP lines. We used the same *Pvcsp* genes, as used for successful complementation of CSP in *P. berghei* (*Pb*) [56], to replace *P. falciparum csp*. These *Pvcsp* genes were amplified from the same plasmids that were used for generation of the *Pb-Pvcsp* lines. In addition, the *Pvcsp* gene coding sequence in the genome of the Pf-PvCSP lines was placed under control of the endogenous *Pfcsp* promoter and transcriptional terminator sequences to ensure correct timing and level of CSP expression. It has been shown that the 3' untranslated region (3'-UTR) of *P. berghei csp* plays an important role in accurate CSP expression, and truncation of *Pbcsp* 3'-UTR results in reduced CSP expression, reduced oocyst sporozoite formation and degeneration of oocysts [57]. The inability of *Pv*CSP to replace the *Pf*CSP function in the chimeric Pf-PvCSP lines is therefore most likely due to sequence differences between these *csp* genes resulting in structural differences of the CSP proteins, which may affect interactions with other parasite proteins that are essential for proper sporozoite formation. These can be protein-interactions that influence correct transport of CSP from within the oocyst-cytoplasm to the membrane of developing sporozoites or protein-interactions that affect CSP localization or maintenance on sporozoites [58-61]. As mentioned above, it is possible to create infectious chimeric *P. berghei* parasites expressing *Pv*CSP. It therefore appears that both *Pv*CSP and *Pf*CSP are able to interact with these CSP interacting proteins in *P. berghei*.

Future studies

Currently, the inability of the two Pf-PvCSP lines to produce salivary gland sporozoites means that these parasites cannot be used for CHMI studies. Further studies are required to investigate if it is possible to create *P. falciparum* parasites that express *P. vivax* CSP molecules; this may consist of creating *P. falciparum* parasites expressing hybrid CSP proteins that contain different domains of *Pf*CSP and *Pv*CSP. Studies using parasites expressing hybrid CSP molecules may also reveal which CSP domains are essential for sporozoite maturation and release and may provide insight into the failure of *Pf*CSP complementation by *Pv*CSP.

While it was not possible to directly create *P. falciparum* parasites where CSP had been replaced with *Pv*CSP, it may be possible to replace in *P. falciparum* other vaccine candidate antigens with the *P. vivax* equivalent, and as such make these parasites suitable for CHMI studies. Finally, it may be possible to create transgenic sporozoites that express the target antigen of both *P. falciparum* and *P. vivax* in the same parasite, and such parasites could be used as an enhanced irradiated or genetically attenuated sporozoite vaccine that could induce protective immune responses against infection with both human malaria parasites.

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APPENDIX

Nedelandse samentvatting

English summary

Acknowledgements

Curriculum vitae

List of publications

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Nedelandse samentvatting

De beschikbaarheid van een verscheidenheid aan transgene knaagdier malariaparasieten die verschillende reportereiwitten tot expressie brengen onder controle van stadium specifieke- of constitutieve promotors, is van grote waarde gebleken in studies naar de functie van genen van de parasiet en in onderzoek gericht op de evaluatie van nieuwe medicijnen en vaccins. De beschikbaarheid van vergelijkbare transgene *Plasmodium falciparum* reporterlijnen zou het onderzoek naar gen-functies en de ontwikkeling van nieuwe behandeltherapieën voor humane parasieten kunnen versnellen. In **Hoofdstuk 2** geven we een overzicht van het gebruik van transgene knaagdier en humane malariaparasieten voor vaccinonderzoek.

In deze thesis beschrijven we een reeks van studies uitgevoerd met *P. falciparum* naar de ontwikkeling van nieuwe CRISPR/Cas9 methodieken ter optimalisatie van de genetische modificatie en het genereren van nieuwe transgene *P. falciparum* reporterparasieten waarmee parasiet-gastheer interacties kunnen worden geanalyseerd en welke toegepast kunnen worden in het onderzoek naar nieuwe medicijnen en vaccins. We hebben ons eerst gericht op het verbeteren van de CRISPR/Cas9 genoommodificatietechniek en het introduceren van transgenen in een nieuw, potentieel neutraal, locus van het *P. falciparum* genoom. Gebruik makend van deze verbeterde CRISPR/Cas9 methodieken werden transgene *P. falciparum* parasieten ontwikkeld die of fluorescente-luminescente reporter eiwitten tot expressie brengen of een potentieel vaccin kandidaat eiwit van *P. vivax*, een andere belangrijke humane malariaparasiet.

In **Hoofdstuk 3** beschrijven we studies gericht op het optimaliseren van genetische modificatie middels CRISPR/Cas9 voor het introduceren van transgenen in het genoom van *P. falciparum*. We rapporteren over het nog verder verbeteren van zowel de CRISPR/Cas9 DNA constructen als de selectieprocedure om zo het genoom van *P. falciparum* sneller en efficiënter te kunnen modificeren en om het mogelijk te maken om transgenen in het genoom in te brengen zonder selectiemarkers. Deze methode is gebruikt voor de stabiele integratie van het gen coderend voor GFP in het *P. falciparum* genoom. Het GFP gen werd geïntroduceerd onder controle van promotors van 3 verschillende *Plasmodium* genen (*calmodulin*, *gapdh* en *hsp70*) om te selecteren voor constitutieve en sterke promotors, die ingezet kunnen worden voor het aansturen van expressie van reporter genen in verschillende levensstadia van de parasiet. Bovenstaande genen werden geselecteerd op basis van hun hoge transcriptie in bloedstadia. We tonen aan dat de *in vitro* groei-kinetiek en medicijn-gevoeligheidsprofielen van de bloedstadia van de in deze studie gegenereerde reporter parasietlijnen (GFP@cam, GFP@gapdh en GFP@hsp70) vergelijkbaar zijn met die van de *P. falciparum* wildtype (NF54) moederlijn. Zowel de seksuele als aseksuele bloedstadia van de drie reporterlijnen lieten GFP fluorescentie zien en flowcytometrische analyse van de fluorescentie intensiteit heeft aangetoond dat de hoogste expressie van GFP plaats vond in schizonten van de GFP@hsp70 lijn.

De verbeterde CRISPR/Cas9 constructen en protocollen zullen er toe bijdragen dat sneller genetisch gemodificeerde *P. falciparum* parasietlijnen ontwikkeld kunnen worden, waaronder lijnen die verschillende reportereiwitten onder verschillende (stadium-specifieke) promotors tot expressie brengen. Bovendien zal deze methode het uitvoeren van opeenvolgende gen-deleties en gen-mutaties vergemakkelijken, wat van waarde kan zijn bij gen functieanalyses en voor het genereren van meervoudig genetische verzwakte parasieten, geschikt voor vaccins.

In bovengenoemde studies voor de introductie in het genoom van de *gfp* expressie-cassettes werd gebruik gemaakt van het *p230p* gen-locus. Omdat dit locus in knaagdier malariaparasieten niet essentieel is, ongeacht het ontwikkelingsstadium van de parasiet, werd verondersteld dat dit gen-locus in de humane malariaparasiet neutraal is (een locus dat gemodificeerd kan worden zonder verandering van het fenotype van de verschillende ontwikkelingsstadia van de levenscyclus). Echter, parasieten waarvan het *p230p* gen-locus was uitgeschakeld waren geheel onverwacht niet langer in staat muggen te infecteren en zich in de mug te ontwikkelen.

In Hoofstuk 4 beschrijven we het fenotype van de *p230p* gen-deletie mutanten (*PfΔp230p*) in muggen en de mogelijke rol van het P230p eiwit in de ontwikkeling van de parasiet in het muggenstadium. P230p behoort tot de kleine familie van zogenaamde s48/45-domain 6-cysteine (6-cys) eiwitten. Twee andere eiwitten die tot deze familie behoren, P48/45 en P230, zijn mede bepalend voor de vruchtbaarheid van gameten en zodanig belangrijke antigeenkandidaten voor transmissie-blokkade vaccins. In tegenstelling tot P48/45 en P230, die in zowel mannelijke als vrouwelijk parasieten tot expressie worden gebracht, komt P230p uitsluitend tot expressie in mannelijke gametocyten en gameten van zowel knaagdier als humane malariaparasieten. De *PfΔp230p* mutanten produceerden normale aantallen mannelijke en vrouwelijke gametocyten waarvan expressie van P48/45 en P230 onveranderd was. Met de activatie van mannelijke gametocyten vindt 'exflagellatie' plaats waarbij de mannelijke gameten worden gevormd. Echter, in tegenstelling tot wild type gameten waren de *PfΔp230p* gameten niet in staat zich aan rode bloedcellen te hechten waardoor de vorming van karakteristieke 'exflagellatie-centra' *in vitro* achterwege bleef. Daarnaast bleek de vorming van zygoten en ontwikkeling van zowel oocysten en sporozoieten in muggen is in afwezigheid van P230p sterk gereduceerd (>98%). Mannelijke gameten van knaagdier malariaparasieten waarvan P230p is uitgeschakeld daarentegen laten een normale binding aan rode bloedcellen zien en de vruchtbaarheid en vorming van oocysten van deze parasieten zijn vergelijkbaar met wildtype parasieten. Deze observaties tonen aan dat *P. falciparum* P230p, net als P230 en P48/45, een sleutelrol speelt in de vorming van zygoten en dat verder onderzoek naar P230p als aangrijppunt voor transmissie-blokkade vaccins zinvol is.

In **Hoofstuk 5** beschrijven we het vervaardigen en evalueren van een *P. falciparum* reporterlijn, die een fusie-eiwit bestaande uit luciferase en mCherry, onder controle van de *etramp10.3* promotor, tot expressie brengt. Deze transgene parasieten werden in kweken van bloed- en leverstadia geanalyseerd, alsmede in muggen. Het *P. falciparum*

ETRAMP10.3 eiwit is gerelateerd aan het UIS4 eiwit van knaagdier malariaparasieten welke ook tot de familie van ETRAMP eiwitten behoort. De promotor van *uis4* wordt toegepast voor de expressie van transgenen in de leverstadia van knaagdier malariaparasieten. Gebruik makend van de in Hoofstuk 3 beschreven CRISPR/Cas9 methodieken werd de *mCherry-luc@etramp10.3* expressie-cassette geïntegreerd in het reeds eerder gekarakteriseerde, neutrale gen-locus *P. falciparum p47* . Deze reporterlijn laat expressie van mCherry zien in gametocyten, sporozoieten en leverstadia, terwijl mCherry niet aangetoond kon worden boven de achtergrond van aseksuele bloedstadia en zich ontwikkelende oocysten. Expressie van luciferase werd aangetoond in aseksuele bloedstadia, gametocyten, sporozoieten en leverstadia met de hoogste expressie gemeten in stadium III-V gametocyten en sporozoieten. Expressie van mCherry en luciferase in gametocyten en sporozoieten maakt deze transgene parasietlijn uitermate geschikt voor het analyseren van de effecten van remmers van de gametocyt-ontwikkeling en het analyseren van de biologie van sporozoieten en leverstadia.

In **Hoofdstuk 6** beschrijven we de ontwikkeling van twee chimere *P. falciparum* parasieten (*Pf-Pv*CSP) waarvan het *csp* gen, met behulp van CRISPR/Cas9 methodieken, werd vervangen door twee *csp* gen varianten (VK210 & VK247) van de humane parasiet *P. vivax*. Als zijnde de belangrijkste oppervlakte-eiwit van sporozoieten, speelt CSP een belangrijke rol in sporozoiet formatie en bij de invasie van de speekselklieren van de mug en levercellen van de gastheer door sporozoieten. Het CSP antigeen is het aangrijpingspunt van het tot nu toe meest geavanceerde malariavaccin (RTS,S) en is ook een zeer belangrijke antigeen voor de ontwikkeling van een vaccin tegen *P. vivax*. Knaagdier malariaparasieten waarvan het *csp* gen werd vervangen door *csp* genen van de humane parasiet *P. falciparum* of *P. vivax* worden gebruikt in preklinische evaluaties van CSP vaccins *in vivo*. Deze chimere knaagdier malariaparasieten produceren sporozoieten in *A. stephensi* muggen welke in staat zijn levercellen van knaagdieren en mensen te infecteren. De beschikbaarheid van chimere *P. falciparum* parasieten waarin het *csp* gen is vervangen door het *csp* gen van *P. vivax* zou mogelijkheden bieden voor het testen van *P. vivax* CSP vaccins in kleinschalige klinische trials gebaseerd op 'controlled human malaria infection' (CHMI) studies. De twee ontwikkelde chimere *Pf-PvCSP* parasietlijnen vertoonden een normale ontwikkeling van aseksuele en seksuele bloedstadia *in vitro* en waren in staat sporozoiet-bevattende oocysten te produceren in *A. stephensi* muggen. We hebben bevestigd dat sporozoieten, afkomstig uit de *Pf-PvCSP* oocysten, de overeenkomstige *Pv*CSP variant tot expressie brengen, maar een groot deel van de oocysten degenereert reeds voordat de sporozoieten volledig tot ontwikkeling zijn gekomen en sporozoieten werden niet aangetoond in de hemocoel noch in de speekselklieren van de mug. In de oocysten van *P. falciparum* waarvan CSP werd uitgeschakeld daarentegen werden geen sporozoieten geproduceerd. Deze observaties tonen aan dat *Pv*CSP de functie van *Pf*CSP deels kan complementeren, maar dat soort-specifieke eigenschappen van CSP noodzakelijk zijn voor het doorlopen van de volledige ontwikkeling van sporozoieten in beide humane malariaparasieten.

English summary

For rodent malaria parasites the availability of a variety transgenic parasite lines expressing different reporter proteins under the control of stage-specific or constitutive promoters have been of great benefit to studies to reveal parasite gene function and to studies focussed on the evaluation of novel drugs and vaccines. The availability of similar transgenic *P. falciparum* reporter lines would also help to advance gene-function studies and studies aiming at development of new therapies for *P. falciparum* parasites. We review the use of transgenic rodent and human parasites for malaria vaccine research in **Chapter 2**.

In this thesis, we describe a set of studies performed in *P. falciparum* to develop novel CRISPR/Cas9 methodologies to improve *P. falciparum* transgenesis and to create novel transgenic reporter parasites that can be used to analyse host-pathogen interactions and for anti-malarial drug and vaccine research. We first focused on improving CRISPR/Cas9 gene editing technologies and the introduction of transgenes into the *P. falciparum* genome using a new potential 'neutral' locus. Using this improved CRISPR/Cas9 methodology, transgenic *P. falciparum* parasites were created that either express fluorescent-luminescent reporters or express a major vaccine candidate from the other major human malaria parasite, *Plasmodium vivax*.

In **Chapter 3** we describe studies aiming at improving CRISPR/Cas9 genetic modification for introduction of transgenes into the genome of *P. falciparum*. We report on further improvements to both the CRISPR/Cas9 transfection constructs and selection protocol to more rapidly modify the *P. falciparum* genome in order to introduce transgenes into the parasite genome without the inclusion of drug-selectable marker genes. This method was used to stably integrate the gene encoding GFP into the *P. falciparum* genome under the control of promoters of three different *Plasmodium* genes (*calmodulin, gapdh* and *hsp70*) to select for constitutive and strong promoters that can be used to drive reporter gene expression. These genes were selected as they are highly transcribed in blood stages. We show that the three reporter parasite lines generated in this study (GFP@cam, GFP@gapdh and GFP@hsp70) have *in vitro* blood stage growth kinetics and drug-sensitivity profiles comparable to the parental *P. falciparum* (NF54) wild-type line. Both asexual and sexual blood stages of the three reporter lines expressed GFP with GFP@hsp70 having the highest fluorescent intensity in schizont stages as shown by flow cytometry analysis of GFP-fluorescence. The improved CRISPR/Cas9 constructs and protocol will aid in the rapid generation of transgenic and modified *P. falciparum* parasites, including those expressing different reporters proteins under different (stage specific) promoters. In addition, this method will make it easier to perform successive gene-deletions and gene-mutations, which will be of value to interrogate parasite gene function and for the development of multiple-attenuated malaria parasites suitable for vaccination. In these studies *gfp*-expression cassettes were introduced into the genome in the *p230p* gene locus. This gene locus was predicted to be a 'neutral' locus (a locus that can be modified without altering the phenotype of the different life cycle stages), since in rodent malaria parasites the gene encoding P230p is dispensable throughout the complete life-cycle. Unexpectedly, disruption of the *P230p* locus in *P. falciparum* created parasites that could not infect and develop in mosquitoes.

In **Chapter 4** we describe the phenotype in mosquitoes of the *p230p* gene-deletion mutants (*PfΔp230p*) and the potential role of the P230p protein in mosquito development. P230p belongs to the small family of so called s48/45 domain 6-cysteine (6-cys) proteins. Two other members of this family, P48/45 and P230, are important for gamete fertility in rodent and human malaria parasites and are leading transmission blocking vaccine antigens. While P48/45 and P230 are expressed in male and female parasites, P230p is expressed only in male gametocytes and gametes in both rodent and human malaria parasites. The *PfΔp230p* mutants produced normal numbers of male and female gametocytes, which retained expression of both P48/45 and P230. Upon activation male *PfΔp230p* gametocytes undergo exflagellation and form male gametes. However, male gametes were unable to attach to red blood cells resulting in the absence of characteristic exflagellation centres *in vitro*. In the absence of P230p, zygote formation as well as oocyst and sporozoite development were strongly reduced (>98%) in mosquitoes. In contrast, male gametes of rodent malaria parasites lacking P230p show normal attachment to red blood cells and have fertilisation rates and oocyst production that is comparable to wild type parasites. These observations demonstrate that *P. falciparum* P230p, like P230 and P48/45, has a vital role in *P. falciparum* male fertility and zygote formation and warrants further investigation as a potential transmission blocking vaccine candidate.

In **Chapter 5**, we describe the creation and evaluation of a *P. falciparum* reporter line that expresses a fusion of mCherry and luciferase driven by the promoter of the *etramp10.3* gene and we examine these transgenic parasites in blood- and liver-stage cultures as well in mosquitoes. The *P. falciparum* ETRAMP10.3 protein is related to the rodent *Plasmodium* UIS4 protein, which is also a member of the ETRAMP protein family. The promoter of the *uis4* gene has been used to drive high transgene expression in liver-stages of rodent malaria parasites. The CRISPR/Cas9 methodology described in **Chapter 3** was used to insert the *mCherry-luc@etramp10.3* expression cassette into the previously characterised 'neutral' *P. falciparum p47* gene locus. This reporter line demonstrates mCherry expression in gametocytes, sporozoites and liver-stages, whereas asexual blood stages and developing oocyst did not show mCherry signals different from background levels. Luciferase expression was demonstrated in asexual blood-stages, gametocytes, sporozoites and liver-stages, with high level reporter expression in stage III-V gametocytes and in sporozoites. The expression of mCherry and luciferase in gametocytes and sporozoites makes this transgenic parasite line a suitable tool to analyse the effect of inhibitors on gametocyte development and to analyse sporozoite and liver-stage biology.

In **Chapter 6** we describe the creation of two chimeric *P. falciparum* parasites (*Pf-Pv*CSP), using CRISPR/Cas9 gene editing methodologies, where the gene encoding circumsporozoite protein (CSP), was replaced by two *csp* gene variants (VK210 and VK247) of the human parasite *P. vivax*. The major sporozoite surface protein CSP plays a critical

role both in sporozoite formation and in sporozoite invasion of mosquito salivary glands and liver cells of the host. CSP is the target antigen of the most advanced *P. falciparum* malaria vaccine (RTS,S) and is also an important vaccine target for *P. vivax*. Rodent malaria parasites where the gene encoding circumsporozoite protein (CSP) has been replaced with *csp* genes from the human malaria parasites, *P. falciparum* or *P. vivax*, are used as pre-clinical tools to evaluate CSP vaccines *in vivo*. These chimeric rodent parasites produce sporozoites in *Anopheles stephensi* mosquitoes that are capable of infecting rodent and human hepatocytes. The availability of chimeric *P. falciparum* parasites where the *P. falciparum csp* gene has been replaced by the *P. vivax csp* would open up possibilities to test *P. vivax* CSP vaccines in small scale clinical trials using controlled human malaria infection (CHMI) studies. The two chimeric *Pf-Pv*CSP lines exhibited normal asexual and sexual blood stage development *in vitro* and produced sporozoite-containing oocysts in *A. stephensi* mosquitoes. We confirmed that the oocyst-derived *Pf-PvCSP* sporozoites express the corresponding *Pv*CSP. However, most oocysts degenerate before sporozoite formation and sporozoites were not found in either the mosquito hemocoel or salivary glands. Unlike the chimeric *Pf-PvCSP* parasites, oocysts of *Pf* parasites lacking CSP expression do not produce sporozoites. Combined our observations show that while *Pv*CSP can partially complement the function of *Pf*CSP, species-specific features of CSP govern full sporozoite maturation and development in the two human malaria parasites.

Acknowledgements

I would like to thank all the wonderful people who contributed to make my thesis possible.

I would like to express my greatest gratitude to my supervisors, **Dr. Shahid Khan** and **Dr. Chris Janse,** for their mentorship, guidance, scientific discussions and patience. I will always be grateful for your support both at the personal level and for having nurtured me to grow as a researcher. My sincerest gratitude goes also to my promoter, **Prof. Dr. Maria Yazdanbakhsh**, for her support throughout my study.

I would like to thank all the members of the **Leiden Malaria Research Group (LMRG): Blandine** and **Séverine** for their personal and scientific support; to **Fiona** for always being motivated to help me with the sometimes hard to love *P. falciparum* parasite. Thanks to **Jai**, **Hans (Hansje)**, **Takashi**, **Shynia, Yukiko, Surendra, Ahmed**, **Aurelie**, **Martha**, **Jingwen** and my students **Niels**, **Rick**, and **Edwin;** I have learnt a lot from all of you. I would like to thank **Meta** and her team: **Beatrice, Marijke, Heleen**, **Munisha**, **Els**, **Roos** and **Carola** for the production of mosquitoes, and standardization of mosquito feedings. I also would like to thank all the collaborators who have contributed to my PhD thesis; **Prof. Robert Sauerwein** from the Radboud University Medical Center and his team: **Marga**, **Geert-Jan**, **Annie**, **Sanne** and **Matthijs.** Thanks go to **Dr. Koen Dechering** from TropiQ and his team: **Angelika, Karin**, **Judith, and Martijn.** I would also like to thank **Prof. Kim Williamson, Dr. Jun Miao, Prof. Liwang Cui, Dr. Jose Rubio** and **Dr. Medhi Ghorbal** for their help during my studies.

Special thanks go to my 'brother' and best friend, **Ahmad Syibli**, who taught me that differences in culture, in religion or other differences are no barrier to a sincere and pure friendship. Thank you so much bro', for making me part of your Malaysian family and for being the best confidant and friend.

I would like to thank the members of my thesis committee, for kindly making time to evaluate my PhD thesis: **Prof. Ron Hokke**, **Prof. Barend Mons**, **Dr. Milly van Dijk** and **Dr. Koen Dechering**.

I also would like to thank all my colleagues of Department of Parasitology in Leiden **Abena, Alwin, Angela, Arifa, Bart, Bruno, Dian, Eric, Eunice, Hermelijn, Leonard, Maria, Mathilde, Michelle, Mikhael, Nikolas, Patrick, Suzanne, Jantien, Corrie, Ulysses, Yoanne** and **Yvonne** for the good times during these years. To the 'other fish' in the aquarium: **Frank**, **Miryam**, **Thiago**, **Ana,** you all made aquarium a very warm and nice place to work.

To my friends, **Mila, Cielo, Ana, Jenny**, **Jazz, Ayda, Roman, Andres, Ago**, thank you so much for always be there for me despite the years that have passed and the distance between us.

My deep and sincere gratitude to my lovely mother **Dennize Edith Marin Mogollon** for being a brave young single mother, thank you for all your love, for all your support, for all the sacrifices you have made in order to give me an education and for all your patience. All that I am, is because of you.

Mis más sinceros agradecimientos a mi abuelita **Edith Mogollon,** gracias por todo tu amor incondicional y por siempre estar ahí para mi. A mi soñadora hermana **Yenniffer,** gracias por tu apoyo y por haber cuidado a mi peque cuando más lo necesitaba. A mis **tías, tíos políticos y primitos**, gracias por haber sido parte de mi crecimiento como mujer, como profesional y como madre. A mi segunda familia, la **familia Alarcón Barrera**, gracias por haberme dado un lugar en su familia y tratarme como una hija más.

Finally, I would like to thank my two beloved men, who are my strength, my centre and my life. To my partner, my love and my best friend **Juan Carlos,** for all your love, patience and strength. For not giving up on me, for leaving your life behind to come to be with us, I thank you. This thesis would not have been possible without you and I am grateful for your support, for your advice and scientific discussions. To my little man and sunshine **Juan Daniel**, thank you so much for making me a better woman, thank you for teaching me what is really important in the life, you arrived and made everything worthwhile, you are my inspiration my peace and happiness. If someday you have the opportunity to read this, remember that you are the best of my life, and all my achievements are for you.

Curriculum vitae

Catherin Yizet Marin Mogollon was born on 30th December 1985, in Bogota, Colombia (South America). She completed a Bachelor's degree in Bacteriology and Clinical Laboratory Science in 2007 at Colegio Mayor University of Cundinamarca. During her Bachelor studies she performed a 12 month internship at the Department of Molecular Biology of Fundacion Instituto de Inmunologia de Colombia (FIDIC), where she gained an interest and experience in molecular biology of the parasites that cause malaria in humans, *Plasmodium falciparum* and *P. vivax*. From 2007 until 2010 she performed her Master's degree studies in Biochemistry at the Universidad Nacional de Colombia, where she completed a Master thesis entitled "Identification, expression and characterization of nicotinamide/mononucleotide adenylyltransferase of *Plasmodium falciparum*" under the guidance of Dr. Maria Helena Ramirez Hernandez. From 2011 until 2012 she worked as a research assistant in the Molecular Biology Laboratory of the Caucaseco Scientific Research Center in Cali, Colombia, on the production of the recombinant protein P48/45 of *P. vivax* as a target for transmission blocking vaccines. From 2012 until 2013 she worked in the Laboratory of Biochemistry at Colegio Mayor de Nuestra Señora del Rosario University (Bogota, Colombia) as a research assistant investigating the relationship between hypoxia and metabolism in cancer cells. In 2013 she was enrolled as a PhD student in the Leiden Malaria Research Group (LMRG) of the Department of Parasitology within the Leiden University Medical Center (LUMC, The Netherlands), with support from a Colciencias-Colfuturo PhD fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). In Leiden she carried out her PhD project under supervision of Dr. Shahid Khan and Dr. Chris Janse. The results of the studies in Leiden are presented in this thesis. After finishing her PhD, Catherin Marin will continue as a post-doc in the Department of Parasitology (LUMC, Leiden) focussing on *P. falciparum* genetic modification, to both study the biology malaria parasites and to develop attenuated parasite vaccines. After this period she intends to return to Colombia and apply the knowledge that she has gained to scientific projects in her country.

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