CHAPTER 5

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

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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.

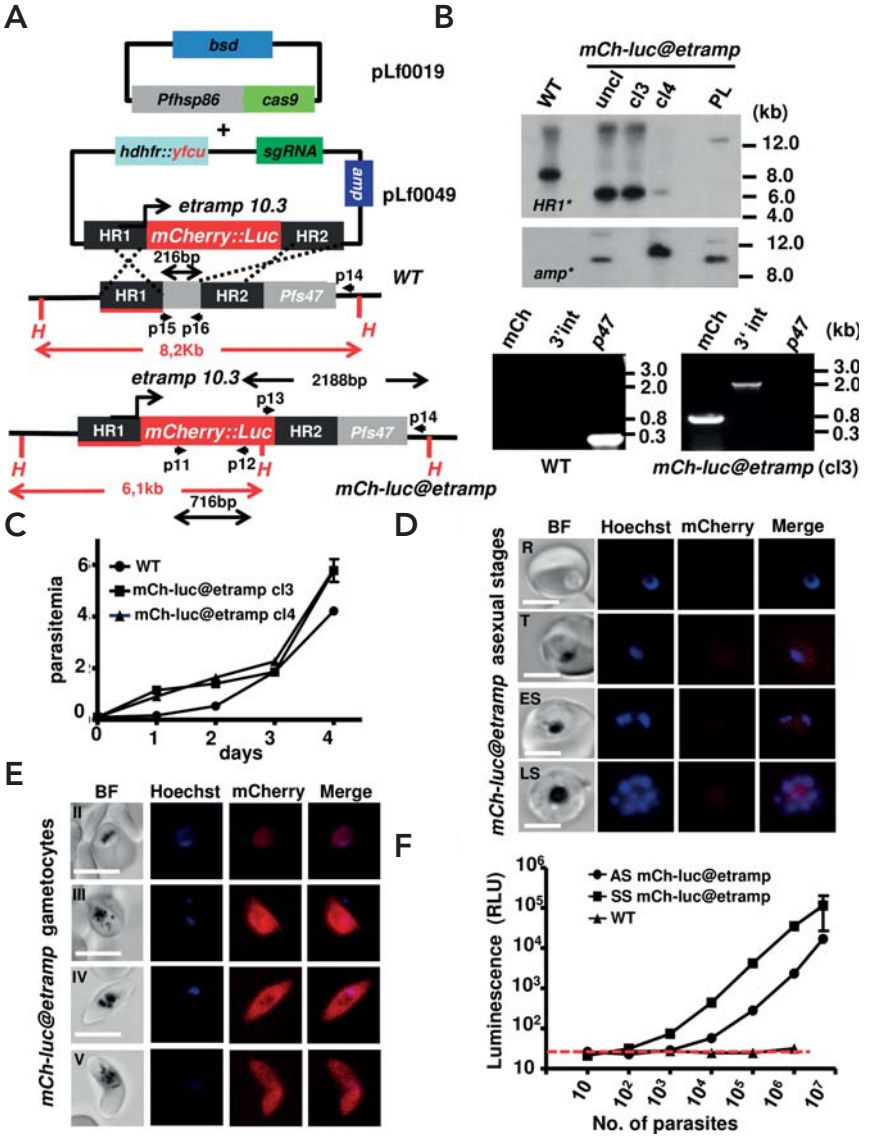

Figure 1. A *P. falciparum* reporter line expressing mCherry-luciferase under control of the *etramp10.3* promoter: generation, genotyping and analysis of expression of mCherry and luciferase in asexual blood stages and gametocytes. **A.** Schematic representation of the Cas9 (pLf0019) and sgRNA/donor (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 *eef1*α 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@eef1*α 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@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 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

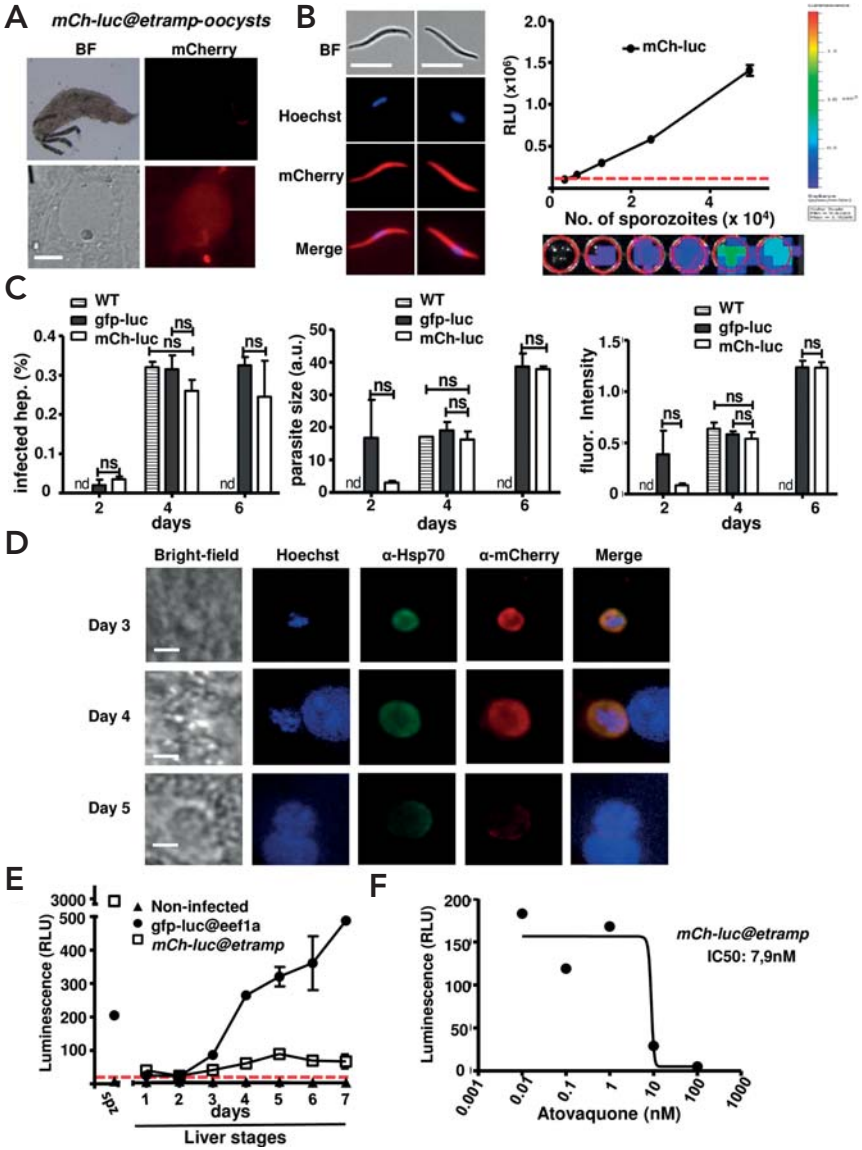

Figure 2. A *P. falciparum* reporter line expressing mCherry-luciferase under control of the *etramp10.3* promoter: analysis of mCherry and luciferase expression in oocyst, sporozoites and liver stages. **A**. Fluorescence microscopy analysis of mCherry expression in live *mcherry-luc@etramp10.3* oocyst 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.

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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.

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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.

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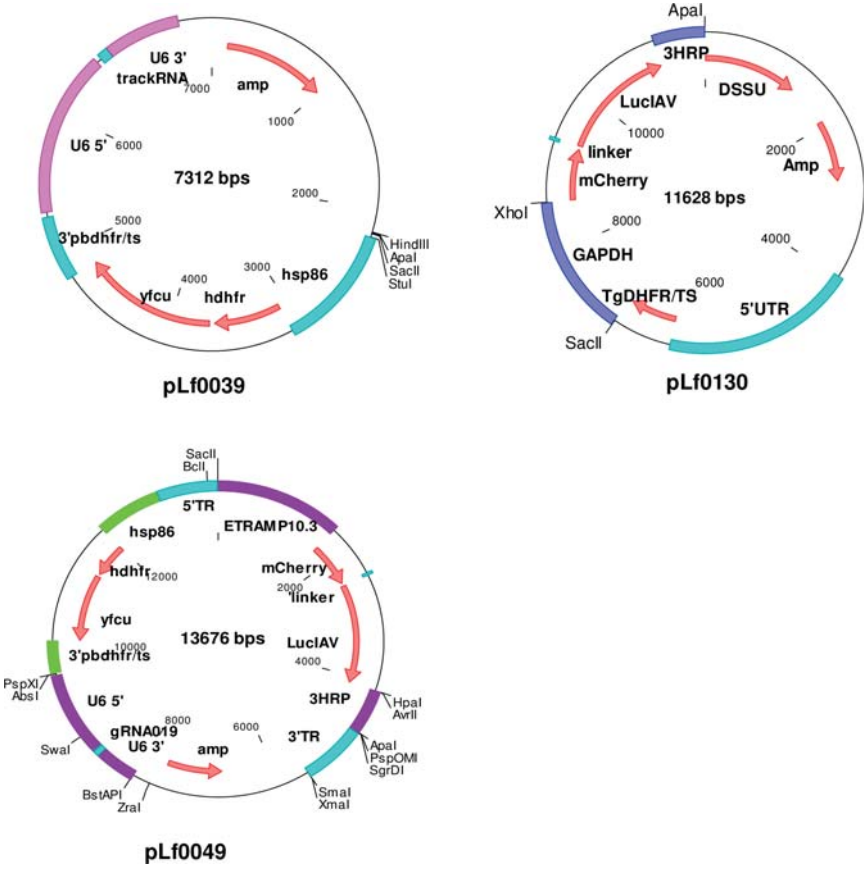
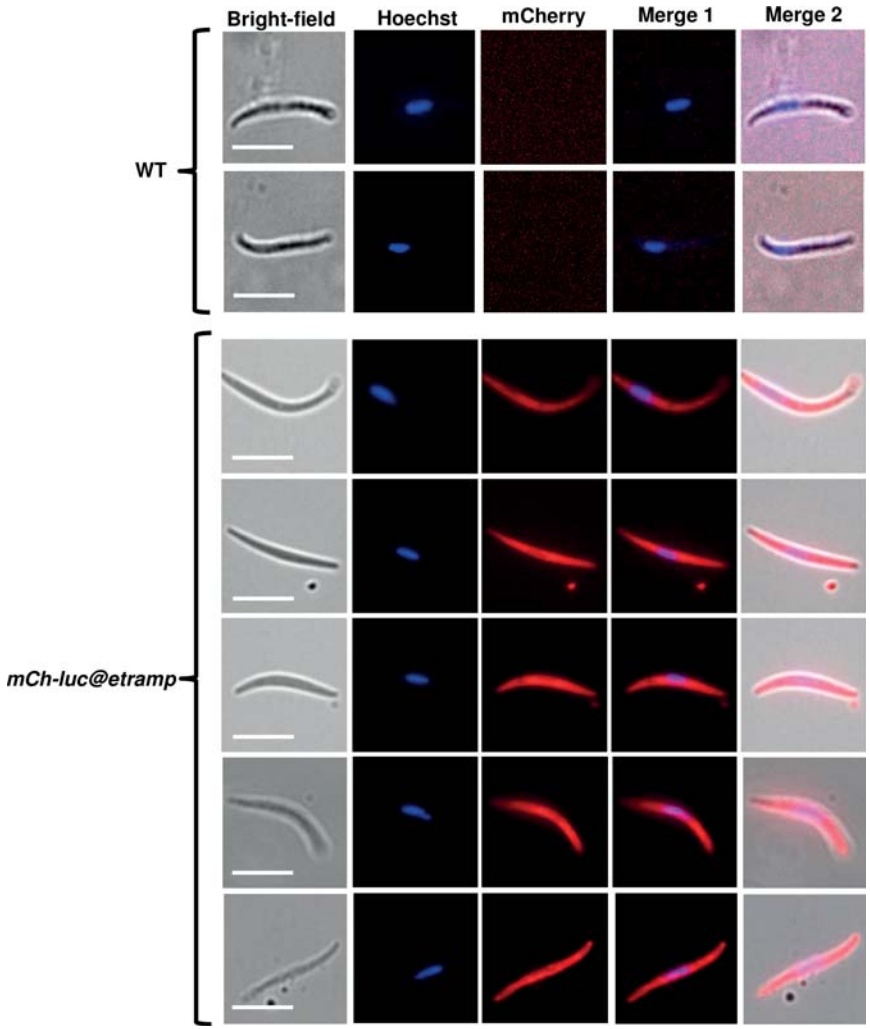
Supplementary Data



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 (x10³)[4]
WT NF54	**range:** Male: 0.5-0.9 Female: 0.9-1.7	**range:** 1000-1700	**range:** 12-60	**Range:** 9-20
mCh-Luc@etramp 0064cl3	Male: 0.6 Female: 1.1 (1 exp.)	**range (SD)** 200-1300 (540) (3 exp.)	**range (SD)** 20-34 (6.6) (3 exp.)	**range (SD)** 8-10 (1.1) (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