# Chapter 2

# Materials and methods

## 2.1. Generation of plasmid constructs

### *2.1.1. Generation of Plasmodium falciparum plasmid constructs*

***Triple-HA (3-HA)-tagged plasmid constructs:*** An approximately 1000bp fragment from the 3' region of the open reading frame of the gene of interest, excluding the STOP codon, was amplified by polymerase chain reaction (PCR) from the genomic DNA of *Plasmodium falciparum* strain 3D7 using the PCR primers (containing the appropriate restriction enzyme sites) listed in Table 2.1. All PCR reactions were performed using KOD Hot Start DNA polymerase (Novagen) in a 50 µL reaction according to manufacturer's instructions. The PCR cycling parameters were as follows:

| Step | Temperature (°C) | Time (min) |
|---|---|---|
| 1 | 95 | 5 |
| 2 | 95 | 1 |
| 3 | 45-58 | 0.5 |
| 4 | 65 | 2 |
| 5 | Go to 2, repeat 35 times | |
| 6 | 65 | 5 |
| 7 | 4 | Forever |

The PCR amplified DNA was digested with the appropriate restriction enzymes overnight at 37°C in a 60 µL reaction. The pCAM-BSD-3HA vector, which allows the endogenous tagging of the gene of interest via single-crossover recombination (Figure 5.1A) [1], was also digested with the same restriction enzymes overnight at 37°C in a 40 µL reaction. All restriction enzymes used were from New England Biolabs (NEB) and restriction digestions were performed according to manufacturer's instructions. The digested PCR amplified insert DNA was collected by ethanol precipitation. The digested pCAM-BSD-3HA vector was resolved by agarose gel electrophoresis using a 1% agarose gel, with electrophoresis performed at a constant voltage of 120V until the DNA was adequately separated, and the required DNA band was gel purified using a QIAquick gel extraction kit (QIAGEN) according to manufacturer's instructions. The digested PCR amplified insert DNA was introduced into the digested pCAM-BSD-3HA vector using $T_4$ DNA ligase (NEB), in a 10 µL ligation reaction, and incubated for 2 hours at 24°C, according to manufacturer's instructions. The products of the ligation reaction were introduced into One Shot® TOP10 chemically competent *E. coli* (Life Technologies) by heat shock for 45 seconds at 42°C, according to manufacturer's instructions. Positive colonies were selected for using LB-agar plates containing 50 µg/mL of ampicillin (Sigma-Aldrich). DNA was extracted from individual colonies using a QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer's instructions, and tested for the presence of the plasmid of interest by restriction

digestion with the appropriate restriction enzymes. DNA containing the desired plasmid was re-introduced into One Shot® TOP10 chemically competent *E.coli* as described above. Single colonies were inoculated into 100 mL of LB broth containing 50 µg/mL ampicillin and grown overnight at 37°C with shaking at 250 rpm. DNA was extracted from the overnight cultures using a GenElute™ HP Plasmid Midiprep Kit (Sigma-Aldrich) and the presence of the desired plasmid again tested by restriction digestion with the appropriate restriction enzymes. A schematic of the pCAM-BSD-3HA plasmid construct is shown in Figure 2.1A.

| Triple-HA-tagged constructs | Primers used (5' - 3') | | Restriction sites | Vector |
|---|---|---|---|---|
| PfMBOAT | Forward | GC**ctgcag**CATGAGGTGTGTTATGAAGCTAGG | *Pst*I and *Hind*III | pCAM-BSD-2HA |
| | Reverse | CCCTCGACTCA**aagctt**TTAAGCGTAATCTGG | | |
| PfDHHC3 | Forward | GC**ctgcag**GGATCGTATAATGATATATCC | *Pst*I and *Xho*I | pCAM-BSD-3HA |
| | Reverse | GCG**ctcgag**ATAATTTTTTAATGTAATTTCTCC | | |
| PfDHHC5 | Forward | GC**ctgcag**CTATACCCACTGATGTTGC | | |
| | Reverse | GCG**ctcgag**TTCAATGTTCATTTCGTTTAATCGC | | |
| PfDHHC7 | Forward | GC**ctgcag**GGAGAACGAAGACATTGTAAATGG | | |
| | Reverse | GCG**ctcgag**TATATTTGTTTTTATTGGAATAATTTCC | | |
| PfDHHC8 | Forward | GC**ctgcag**GATTGGTAATTGTGTAGGAAATCG | | |
| | Reverse | GCG**ctcgag**GAGCGTTTTGAAAATGTTAATATATTTTTGAC | | |
| PfDHHC9 | Forward | GC**ctgcag**CCGATTTTTATAAATGCTCTTTGTGCC | | |
| | Reverse | GCG**ctcgag**ATCTCCATCTTCCTTTATGTTTTC | | |

**Table 2.1: Primers used for the generation of *P. falciparum* triple-HA-tagged plasmid constructs.** The restriction enzyme sites are highlighted in red and indicated, along with the vector used, in the columns on the right.

***Double-crossover knock-out plasmid constructs:*** Generation of the double-crossover knock-out plasmid constructs were performed essentially as described above, except that an approximately 600-800bp fragment from both the N-terminal and C-terminal regions of the open reading frame of the gene of interest was amplified by PCR and introduced, one fragment at a time, into multiple cloning site 1 and multiple cloning site 2 of the pCC1 vector respectively, which allows the disruption of a gene of interest by double-crossover recombination (Figure 5.4A) [2]. PCR cycling parameters were as described above and the restriction enzymes used are as described in Table 2.2. In this case, ligation reactions were performed in 20 µL volumes and left overnight at 24°C, after which the products of the ligation reactions were collected by ethanol precipitation, before being introduced into competent cells. A schematic of the double-crossover knock-out plasmid construct is shown in Figure 2.1B.

| Knock-out constructs | Fragment | Primers used (5' - 3') | | Restriction sites | Vector |
|---|---|---|---|---|---|
| PfMBOAT | Nt | Forward | GC**ccgcgg**GAATAATAAAAATGATGAGGGAC | *Sac*II and *Spe*I | pCC-1 |
| | | Reverse | GCG**actagt**CATAGCTATAAATTGAGGGTGG | | |
| | Ct | Forward | GC**gaattc**CCATTAAAATTAGGTTTTACCGG | *Eco*RI and *Avr*II | |
| | | Reverse | GCG**cctagg**CACTTTTGAGATATGTGTCCATGGTGG | | |
| PfDHHC3 | Nt | Forward | GC**ccgcgg**GCTTATAATATAATCCCAAATTTTCC | *Sac*II and *Spe*I | |
| | | Reverse | GCG**actagt**CACATTTATTACAGACTTTACAGTGC | | |
| | Ct | Forward | GC**gaattc**CCTTAATACATCTCACCAC | *Eco*RI and *Nco*I | |
| | | Reverse | GCG**ccatgg**CCTCTTTCAATTTCGTGTTTTTCC | | |
| PfDHHC5 | Nt | Forward | GC**ccgcgg**CAACATCATGTGTGTCCATTAACG | *Sac*II and *Spe*I | |
| | | Reverse | GCG**actagt**GCTGCCCAATCAATTACCGTAC | | |
| | Ct | Forward | GC**gaattc**GTATGGGTATTGAAAATGCTAG | *Eco*RI and *Avr*II | |
| | | Reverse | GCG**cctagg**CTTATAATTACACTTAATAAATTACCAC | | |
| PfDHHC7 | Nt | Forward | GC**ccgcgg**GACAAAGAATAAGAATGTTGAAG | *Sac*II and *Spe*I | |
| | | Reverse | GCG**actagt**CCTTATAAATTAAAGGCAAACAGTG | | |
| | Ct | Forward | GC**gaattc**CGTTCTTATCCCTCATAGTAACATG | *Eco*RI and *Nco*I | |
| | | Reverse | GCG**ccatgg**GTTTTTATTGGAATAATTTCCTCTACTG | | |
| PfDHHC8 | Nt | Forward | GC**ccgcgg**GGTTCAAATACATGATGCAAAATCTCC | *Sac*II and *Spe*I | |
| | | Reverse | GCG**actagt**CCAGGGTCACAAAATGCTGTAG | | |
| | Ct | Forward | GC**gaattc**CCTTTCCATCCTCCCTTTTG | *Eco*RI and *Nco*I | |
| | | Reverse | GCG**ccatgg**GCGTTTTGAAAATGTTAATATATTTTTG | | |
| PfDHHC9 | Nt | Forward | GC**ccgcgg**GAATAATTATTTGGCATTTATC | *Sac*II and *Spe*I | |
| | | Reverse | GCG**actagt**CCTGCAGTGATGAGCTCTCTCTGG | | |
| | Ct | Forward | GC**ccatgg**cgctatctcttttagATGAACTGTC | *Eco*RI and *Avr*II | |
| | | Reverse | GCG**cctagg**CTCCATCTTCCTTTATGTTTTC | | |

**Table 2.2: Primers used for the generation of *P. falciparum* double-crossover knock-out plasmid constructs.** The restriction enzyme sites are highlighted in red and indicated, along with the vector used, in the columns on the right.

### *2.1.2. Generation of Plasmodium berghei plasmid constructs*

*P. berghei* triple-HA-tagging and knock-out targeting vectors were obtained from the open access *Plasmo*GEM resource hosted at the Wellcome Trust Sanger Institute (http://plasmogem.sanger.ac.uk). Construction of the *Plasmo*GEM vectors are as previously described [3], and the transfection of these vectors into *P. berghei* strain ANKA 2.34 purified schizonts by electroporation was performed as previously described [4]. All *P. berghei* transfection procedures and the extraction of blood from infected mice were kindly performed by Ellen Bushell, Billker and Rayner labs. The *Plasmo*GEM vectors used here are listed in Table 2.3.

| PbDHHC | *Plasmo*DB Gene names | Vector Design | *Plasmo*GEM transfection vector names |
|---|---|---|---|
| PbDHHC3 | PBANKA_092730 | TAG | PbGEM-094114 |
| | | KO | PbGEM-111866 |
| PbDHHC4 | PBANKA_142090 | TAG | PbGEM-065194 |
| | | KO | PbGEM-065186 |
| PbDHHC5 | PBANKA_133780 | TAG | PbGEM-058319 |
| | | KO | PbGEM-072266 |
| PbDHHC6 | PBANKA_083330 | TAG | PbGEM-112088 |
| | | KO | PbGEM-027807 |
| PbDHHC7 | PBANKA_124300 | TAG | PbGEM-052430 |
| | | KO | PbGEM-104070 |
| PbDHHC8 | PBANKA_141970 | TAG | PbGEM-065002 |
| | | KO | PbGEM-225987 |
| PbDHHC9 | PBANKA_093210 | TAG | PbGEM-121234 |
| | | KO | PbGEM-121226 |
| PbDHHC10 | PBANKA_051200 | TAG | PbGEM-112097 |
| | | KO | PbGEM-015165 |
| PbDHHC11 | PBANKA_031260 | TAG | PbGEM-225995 |
| | | KO | PbGEM-121242 |

**Table 2.3: PlasmoGEM vectors used for the generation of 3HA-tagged and knock-out transgenic strains in *P. berghei.***

### *2.1.3. Generation of Human Embryonic Kidney 293E (HEK293E) expression plasmid constructs*

The sequences coding for the *P. falciparum* proteins of interest were codon-optimised for expression in Human embryonic kidney 293 (HEK293) cells using the *GeneART* gene synthesis service (Life Technologies). *Plasmodium* parasites lack the enzymatic machinery required for the N-linked glycosylation of proteins, thus all *P. falciparum* proteins lack N-linked glycosylation moieties. All potential N-linked glycosylation sites (N-X-S/T) were therefore removed from the *P. falciparum* proteins of interest by substituting the serine/threonine residues at these sites with alanine. The sequence coding for either the FLAG® tag or the c-Myc tag was added to the C-terminal region of the proteins of interest, followed by a STOP codon. The entire sequence of the protein of interest, including the FLAG/c-Myc tag and the STOP codon, was then flanked by unique NotI (5'-end) and AscI (3'-end) restriction sites in order to introduce the sequences into a pTT3-based expression vector, which also contains a region coding for the immunoglobulin-like domains 3 and 4 of rat CD4 [5], using the *GeneART* gene synthesis service. As CD4 was not required in this case, the STOP codon was introduced directly after the FLAG or c-Myc tag, upstream of the CD4 region. A schematic of the HEK293 expression construct containing the c-Myc/FLAG-tagged codon-optimised *P. falciparum* gene of interest is shown in Figure 2.1C.

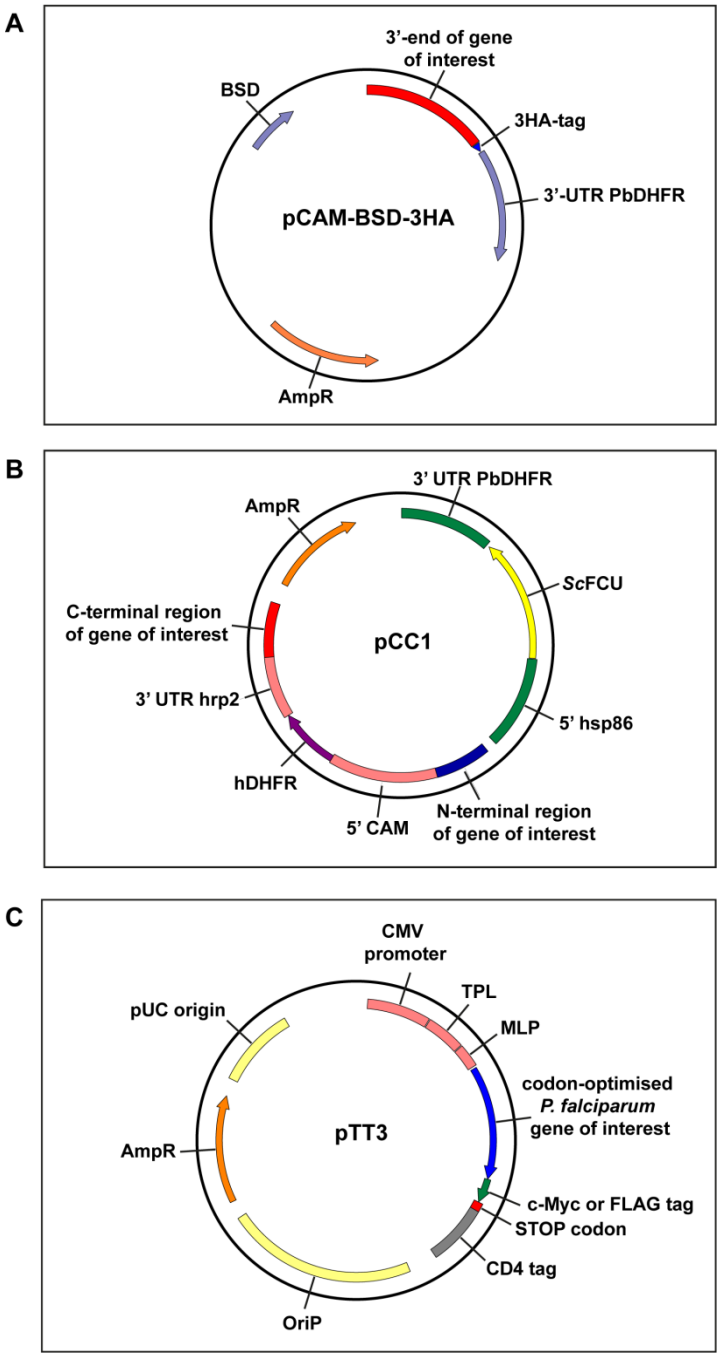


**Figure 2.1: Schematic of the *P. falciparum* and HEK293E expression plasmid constructs. (A)** Schematic of the pCAM-BSD-3HA plasmid construct used for the C-terminal 3-HA-tagging of *P. falciparum* genes of interest. 3'UTR PbDHFR - 3' untranslated region of the *P. berghei* dihydrofolate reductase, AmpR - ampicillin resistance gene. **(B)** Schematic of the pCC1 plasmid construct used to knock-out *P. falciparum* genes of interest via double-crossover homologous recombination. 5' CAM - calmodulin promoter, hDHFR - human dihydrofolate reductase gene, 3' UTR hrp2 - 3' untranslated region of *P. falciparum* hrp2 gene, 5' hsp86 - *P. falciparum* hsp86 promoter, *Sc*FCU - chimaeric *S. cerevisiae* cytosine deaminase and uracil phosphoribosyl transferase, 3' UTR PbDHFR - 3' untranslated region of *P. berghei* dihydrofolate reductase, AmpR - ampicillin resistance gene. **(C)** Schematic of the pTT3-based HEK293 expression plasmid construct used for the expression of codon-optimised *P. falciparum* genes of interest in HEK293 cells. CMV promoter - cytomegalovirus promoter, TPL - tripartite leader sequence, MLP - adenovirus major late promoter enhancer, OriP - Epstein-Barr virus origin of replication, AmpR - ampicillin resistance gene, pUC origin - bacterial origin of replication.

*2.1.4. Site-directed mutagenesis and point mutations of HEK293 expression plasmid constructs*

***PfDHHC5_CdA:*** In order to mutate the cysteine residue within the DHHC domain of PfDHHC5 into an alanine residue, the *Gene*ART-synthesized expression plasmid coding for PfDHHC5 was subjected to site-directed mutagenesis using the primers listed in Table 2.4 and the QuikChange II XL Site Directed Mutagenesis Kit (Agilent Technologies), according to manufacturer's instructions. Primers were designed using the free online tool, QuikChange Primer Design Program (Agilent Technologies). DNA containing the desired mutation was introduced into One Shot® TOP10 chemically competent *E.coli* as described above. Single colonies were inoculated into 100 mL of LB broth containing 50 µg/mL ampicillin and grown overnight at 37°C with shaking at 250 rpm. DNA was extracted from the overnight cultures using a GenElute™ HP Plasmid Midiprep Kit (Sigma-Aldrich) according to manufacturer's instructions, and used for the transient transfection of HEK293 cells (described below).

***PfSec22 and PfARO:*** In order to mutate the cysteine residues of interest in PfSec22 and PfARO, the primers listed in Table 2.4 (which contain the desired cysteine to alanine point mutations) were used to amplify the genes of interest by PCR, using the *Gene*ART-synthesized expression plasmids as templates. PCR conditions and cycling parameters are as previously described above. The amplified DNA was restriction digested using the restriction enzymes listed in Table 2.4, and re-introduced into the *Gene*ART expression plasmids using $T_4$ DNA ligase, following the methods previously described above. DNA containing the desired mutation was introduced into One Shot® TOP10 chemically competent *E.coli* as described above. Single colonies were inoculated into 100 mL of LB broth containing 50 µg/mL ampicillin and grown overnight at 37°C with shaking at 250 rpm. DNA was extracted from the overnight cultures using a GenElute™ HP Plasmid Midiprep Kit (Sigma-Aldrich) and used for the transient transfection of HEK293 cells (described below).

| Site-directed mutagenesis construct | Primers (5'-3') | | Restriction sites | Vector |
|---|---|---|---|---|
| PfDHHC5-CdA | Sense | GAAATCTTCGACCACCAC**GCC**GACTTCACCCTGAACTG | - | pTT3-CD4 expression vector |
| | Antisense | CAGTTCAGGGTGAAGTC**GGC**GTGGTGGTCGAAGATTTC | | |
| PfSec22-C2dA | Forward | GC**gcggccgc**ATG**GCC**GATGTGGTGCTGCTGTGCAG | *Not*I and *Asc*I | |
| | Reverse | GCG**ggcgcgcc**TCAGAGATCCTCTTCAGAAATCAGC | | |
| PfARO-C5dA | Forward | GC**gcggccgc**ATGGGCAACAAT**GCC**TGCGCCGGC | *Not*I and *Asc*I | |
| | Reverse | GCG**ggcgcgcc**TCAGAGATCCTCTTCAGAAATCAGC | | |
| PfARO-C6dA | Forward | GC**gcggccgc**ATGGGCAACAATTGC**GCC**GCCGGC | *Not*I and *Asc*I | |
| | Reverse | GCG**ggcgcgcc**TCAGAGATCCTCTTCAGAAATCAGC | | |

**Table 2.4: Primers used for the generation of point mutations in HEK293 expression plasmid constructs.** The codon targeted for the point mutation is underlined and highlighted in red. The restriction enzyme sites are highlighted in red and indicated along with the vector used in the columns on the right.

## 2.2. *Plasmodium in vitro* cell culture and transfection

### *2.2.1. Plasmodium falciparum culture*

*Plasmodium falciparum* strain 3D7 was maintained in 'complete' media consisting of RPMI 1640 media (Life Technologies) supplemented with 30 mM HEPES (Sigma-Aldrich), 0.05 mg/mL hypoxanthine (Sigma-Aldrich), 0.025 mg/mL Gentamicin (Sigma-Aldrich), 2 mg/mL glucose (Sigma-Aldrich), 0.24% sodium bicarbonate (Sigma-Aldrich), and 10% $O^+$ heat-inactivated human serum or 0.5% AlbuMAX® II (Life Technologies), along with $O^+$ human erythrocytes at 5% haematocrit. All cultures were gassed with 5% $CO_2$ and 0.5% $O_2$ in $N_2$, according to established methods [6].

### *2.2.2. Synchronisation of P. falciparum parasites by sorbitol lysis*

The pellet from *P falciparum*-infected blood-stage culture was collected by centrifugation at 800xg for 5 minutes with low breaks and resuspended in 5 volumes of 5% sorbitol (Sigma-Aldrich) in water. The suspension was left standing at room temperature for 5 minutes and was subjected again to centrifugation in order to remove the sorbitol. The pellet was washed once in RPMI before being resuspended in complete media and put back into culture.

*2.2.3. Transfection of Plasmodium falciparum*

Transfection of ring-stage 3D7 parasites was performed according to published protocols [7]. Approximately 100-150 µg of DNA to be transfected was collected by ethanol precipitation and fully resuspended in 40 µL of sterile 1xTE buffer. Incomplete cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM $K_2HPO_4/KH_2PO_4$ pH 7.6, 25 mM HEPES/2 mM EGTA pH 7.6, 5 mM $MgCl_2$) was added to the DNA, to a total volume of 400 µL, and the DNA/incomplete cytomix mixture placed on ice. The pellet from 5 mL of *P. falciparum in vitro* blood-stage culture (at 5% haematocrit with ring stage parasites at 8-12% parasitemia) was collected by centrifugation at 800xg for 5 minutes with low brakes and washed in 5 mL of incomplete cytomix. The pellet was then resuspended in the DNA/incomplete cytomix mixture and transferred into the bottom of a cold 2 mm electroporation cuvette (Bio-Rad). The entire mixture was electroporated using the Gene Pulser Xcell™ Electroporation System (Bio-Rad) with the following settings: 0.31 kV, 950 µF. The transfected cells were washed with 10 mL of RPMI media and then resuspended in 10 mL of pre-warmed complete media with 0.5 mL of freshly washed erythrocytes (50% haematocrit), and returned to culture. One day post-transfection, positive drug selection was started using either 2.5 µg/mL Blasticidin-S (Fisher Scientific) or 2.5 nM WR99210. Drug selection was maintained until stable parasite growth was obtained, which normally occurred 14 to 25 days post-selection. In order to select for parasites containing the construct integrated via homologous recombination, drug cycling was performed, where the transfected parasites were grown without drug pressure for 3 weeks, after which drug pressure was reapplied until stable parasite growth was once again attained. For knock-out strains transfected with the pCC1 plasmid, after drug cycling, negative selection was then performed by adding 1 µM 5-fluorocytosine (5FC) (Sigma-Aldrich), in the presence of 2.5 nM WR99210, in order to select for parasites which had integrated the construct via double cross-over recombination, and to select against parasites still harbouring the episomal plasmid. This negative selection was maintained until stable parasite growth was obtained [2].

*2.2.4. Cloning by limiting dilution*

*P. falciparum* blood-stage culture was diluted in complete media and added into the wells of a round-bottomed 96-well plate at concentrations of 0.5 parasites/well and 1 parasite/well. A concentration of 3 parasites/well was also included in two rows of the 96-well plate, as a positive control for the limiting dilution. Freshly washed erythrocytes were added into each well until a 2% haematocrit was achieved, and the volume in each well was then brought up to a total of 100 µL. Media was changed to selective media (complete media containing 2.5 µg/mL Blasticidin-S or complete media containing

2.5 nM WR99210) 10-12 days later. Smears were taken from each of the wells on day 14 up to day 21 until parasites were seen. Approximately 10-15 clones were selected, preferably from wells with higher dilutions, and gradually raised to 10 mL of complete media at 5% haematocrit.

### *2.2.5. Saponin lysis of P. falciparum infected erythrocytes*

The pellet from *P. falciparum*-infected blood-stage culture was collected by centrifugation at 800xg for 5 minutes with low brakes. The pellets were resuspended in 10 volumes of 0.1% saponin in PBS and left at room temperature for 10 minutes to allow the lysis of erythrocytes to occur. Parasite material was collected by centrifugation at 3220xg for 10 minutes and the supernatant containing lysed erythrocyte material was discarded. The resultant parasite pellet was repeatedly washed with 0.1% saponin/PBS until the supernatant was clear. The supernatant from the final wash was removed and the saponin pellet frozen at -80°C until use. For SDS-PAGE and Western blot analysis (described in Section 2.5.1), the saponin pellet was lysed in 4% SDS/50 mM Tris-Cl pH 7.4/150 mM NaCl/5 mM EDTA in the appropriate volume which would result in a concentration of $5x10^8$ parasites/mL. Lysis was allowed to occur for 30 minutes at 37°C with shaking. Any insoluble material was removed by centrifugation at 20238xg for 5 minutes and 10-20 µL of the resultant supernatant was diluted in 2X Laemmli sample buffer (4% SDS, 120 mM Tris-Cl pH 6.8, 20% glycerol, 0.02% bromophenol blue) with 10% β-mercaptoethanol (Sigma-Aldrich) and analysed as described in Section 2.5.1.

### *2.2.6. P. falciparum growth assay*

*In vitro* blood-stage culture infected with *P. falciparum* transgenic strains of interest, as well as with wild-type 3D7 strain as a control, was diluted appropriately to produce a suspension of 2% haematocrit and 1% parasitemia. A volume of 100 µL of each suspension was added into each well of a round-bottomed 96-well plate (labelled 'Day 0') and the plate incubated for 2 days under the standard *P. falciparum* culture conditions described in Section 2.2.1. Additionally, 10 µL of each parasite suspension was also fixed in 50 µL of fixative (2% formaldehyde/0.2% gluteraldehyde/PBS) for 45 minutes at 4°C. The fixed cells were collected by centrifugation at 450xg for 2 minutes and washed with PBS. These fixed cells were named 'Day 0' and kept in 50 µL PBS until further use. After the 2 day incubation period, 10 µL aliquots from each well of the 'Day 0' 96-well plate were taken into a new 96-well plate (labelled 'Day 2') and fixed in 50 µL per well of fixative as described above. These fixed cells, along with the previously fixed 'Day 0' cells, were then permeabilised in 50 µL per well of 0.3% Triton X-100/PBS for 10 minutes at 24°C, followed by a wash with PBS. The cells were

then incubated in 50 µL/well of ribonuclease A (MP Biomedicals), at a concentration of 0.5 mg/mL, for 45 minutes at 37°C, followed by another wash with PBS. Finally, the cells were stained with 50 µL/well of SYBR® Green I (Invitrogen)/PBS, at a concentration of 1:5000, for 45 minutes at 37°C. The stained cells were washed twice with PBS and were then resuspended in 200 µL/well of PBS. Parasitemia of the samples from 'Day 0' and 'Day 2' was then counted by flow cytometry as previously described [8], with kind assistance from Michel Theron, Rayner lab. The cultures in the original 'Day 0' plate were then diluted 1:5 or 1:10 as appropriate and incubated for a further 2 days under the standard *P. falciparum* culture conditions. Samples were again taken two days later (cells collected were then labelled 'Day 4') and the cells fixed and stained as described above. The cultures in the original 'Day 0' plate were again diluted 1:5 or 1:10 accordingly. This was repeated every two days until 'Day 10' samples were collected, fixed and stained. Each sample for every transgenic strain tested was performed in triplicate.

### *2.2.7. Purification of P. berghei schizonts*

*P. berghei*-infected mouse blood was kindly provided by Ellen Bushell, Billker lab, and added into 50 mL of schizont culture media, which consisted of RPMI 1640 (supplemented with 25 mM HEPES and 2 mM L-Glutamine) (Sigma-Aldrich), 24 mM sodium bicarbonate pH 7.2 (Sigma-Aldrich), 25% heat-inactivated foetal bovine serum (FBS) and 1:100 Penicillin/Streptomycin (Life Technologies). The schizont culture was gassed (5% $CO_2$ and 0.5% $O_2$ in $N_2$) and incubated overnight at 37°C with shaking. The red blood cell pellet was then collected by centrifugation at 180xg for 15 minutes with low acceleration/brakes. The supernatant was removed and the pellet was layered on top of a Nycodenz gradient (2.75 mL of Nycodenz stock in 2.25 mL of PBS) and subjected to centrifugation at 300xg for 30 minutes with low acceleration/brakes. The schizont-containing interlayer was then removed and washed with schizont culture media. The purified schizont pellet was collected from the wash by centrifugation at 300xg for 8 minutes with low acceleration/brakes. For immunofluorescence assays, 1-2 µL of the purified schizonts were resuspended in 500 µL FBS, and 100 µL of the schizont/FBS suspension was dropped onto poly-l-lysine coverslips (BD Biosciences) and was analysed as described in Section 2.5.2. For SDS-PAGE and Western blot analysis, the purified schizont pellet was resuspended in 30-50 µL of 2X Laemmli sample buffer (4% SDS, 120 mM Tris-Cl pH 6.8, 20% glycerol, 0.02% bromophenol blue) with 10% β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 minutes at 95°C, followed by analysis as described in Section 2.5.1.

## 2.3. Genotyping of transgenic parasite strains

### *2.3.1. Genotyping by PCR*

Genomic DNA was extracted from *in vitro* blood-stage culture infected with *P. falciparum* transgenic triple-HA-tagged or knock-out strains of interest, or the wild-type 3D7 strain as a control, using the QIAamp DNA Blood Mini Kit (QIAGEN) according to manufacturer's instructions. The genomic DNA extracted for each transgenic strain was genotyped using the specific primers listed in Table 2.5 and Table 2.6. The PCR cycling parameters were as described in Section 2.1.1.

| Triple-HA-tagged line | Primer | Sequence (5'-3') |
|---|---|---|
| PfDHHC3 | P1 | GTAGGATTGTTATCTACCTTAACCC |
| | P2 | CTCGATATTATTAAACAAGCCGATC |
| | P3 | GAACATATTTATTAAACTGCAG |
| | P4 | GCTATTTACATGCATGTGCATGCAC |
| | P5 | CACATTTGTGCATTTAGAGATGATAC |
| PfDHHC5 | P1 | GTTGCCCATATTTCTTAGCATTTATAC |
| | P2 | CAATGTTCATTTCGTTTAATCGC |
| | P3 | GAACATATTTATTAAACTGCAG |
| | P4 | GCTATTTACATGCATGTGCATGCAC |
| | P5 | GAAGCAAATGGTACTATAGGCCC |
| PfDHHC7 | P1 | CCAGAGGAATTATTGAGATGGGGG |
| | P2 | CATATAAAATGCATATTATTCAAGCAG |
| | P3 | GAACATATTTATTAAACTGCAG |
| | P4 | GCTATTTACATGCATGTGCATGCAC |
| | P5 | GGATACGTCAAATAATGTGATGTTGG |
| PfDHHC8 | P1 | GGAATTATGTTGATTTATCATTGCC |
| | P2 | CGAGGTTTATGATCCCGCTGCCG |
| | P3 | GAACATATTTATTAAACTGCAG |
| | P4 | GCTATTTACATGCATGTGCATGCAC |
| | P5 | - |
| PfDHHC9 | P1 | CCATGGATAGGCACTTGTGTAGG |
| | P2 | CTCCATCTTCCTTTATGTTTTC |
| | P3 | GAACATATTTATTAAACTGCAG |
| | P4 | GCTATTTACATGCATGTGCATGCAC |
| | P5 | CGATTCAGACTTGTAATAAGTGC |
| PfMBOAT | P1 | GATACTGAACCCACACTTTCATC |
| | P2 | CCATGGTGGTAAAATATATGCACGTG |
| | P3 | GAACATATTTATTAAACTGCAG |
| | P4 | GCTATTTACATGCATGTGCATGCAC |
| | P5 | GAATAATAAAAATGATGAGGGAC |

**Table 2.5: Primers used for the genotyping of *P. falciparum* triple-HA-tagged lines.**

| Knock-out line | Primer | Sequence (5'-3') |
|---|---|---|
| PfDHHC3 | P6 | GAACGTATAATCTTTATAAAGTTTTGCC |
| | P7 | CCTCTTTCAATTTCGTGTTTTTCC |
| | P8 | GGAATACTAAATATATATCCAATGGCCCC |
| | P9 | GCGACGATGCAGTTTAGCGAACC |
| | P10 | GGGTTAAGGTAGATAACAATCCTAC |
| | P11 | - |
| | P12 | GTAGGATTGTTATCTACCTTAACCC |
| | P13 | CCATATGCGGTGTGAAATACCGC |
| PfDHHC5 | P6 | GTTTATCCTTTAACCTTTTTATAGTATG |
| | P7 | CTTATAATTACACTTAATAAATTACCAC |
| | P8 | GGAATACTAAATATATATCCAATGGCCCC |
| | P9 | GCGACGATGCAGTTTAGCGAACC |
| | P10 | GTCATCCGTTCCTATGTCGTAAAC |
| | P11 | GTTTTCACAGTATTGAAGTTTAATTTGC |
| | P12 | GTTTACGACATAGGAACGGATGAC |
| | P13 | CCATATGCGGTGTGAAATACCGC |
| PfDHHC7 | P6 | GATGTATAATAAAAGACGAATGAAGTGTC |
| | P7 | GTTTTTATTGGAATAATTTCCTCTACTG |
| | P8 | GGAATACTAAATATATATCCAATGGCCCC |
| | P9 | GCGACGATGCAGTTTAGCGAACC |
| | P10 | CCATTTACAATGTCTTCGTTCTCC |
| | P11 | - |
| | P12 | GGAGAACGAAGACATTGTAAATGG |
| | P13 | CCATATGCGGTGTGAAATACCGC |
| PfDHHC8 | P6 | GTTCTCAACAGCAGTAATACATAC |
| | P7 | GCGTTTTGAAAATGTTAATATATTTTTG |
| | P8 | GGAATACTAAATATATATCCAATGGCCCC |
| | P9 | GCGACGATGCAGTTTAGCGAACC |
| | P10 | GTATAACAATGTTTACTTCTAGGTTC |
| | P11 | - |
| | P12 | GAACCTAGAAGTAAACATTGTTATAC |
| | P13 | CCATATGCGGTGTGAAATACCGC |
| PfDHHC9 | P6 | CAATTATGCAATATGTTGTATAAATG |
| | P7 | CTCCATCTTCCTTTATGTTTTC |
| | P8 | GGAATACTAAATATATATCCAATGGCCCC |
| | P9 | GCGACGATGCAGTTTAGCGAACC |
| | P10 | GGCACAAAGAGCATTTATAAAAATCGG |
| | P11 | GAAAATAATGGAATGTTAAATTAGGAAATAC |
| | P12 | CCGATTTTTATAAATGCTCTTTGTGCC |
| | P13 | CCATATGCGGTGTGAAATACCGC |
| PfMBOAT | P6 | GTGTGTTTCGTAATATTCACTTTTTAG |
| | P7 | CACTTTTGAGATATGTGTCCATGGTGG |
| | P8 | GGAATACTAAATATATATCCAATGGCCCC |
| | P9 | GCGACGATGCAGTTTAGCGAACC |
| | P10 | GTGTATGAATATTATGAGCACACG |
| | P11 | - |
| | P12 | CGTGTGCTCATAATATTCATACAC |
| | P13 | CCATATGCGGTGTGAAATACCGC |

**Table 2.6: Primers used for the genotyping of *P. falciparum* knock-out lines.**

*2.3.2. Reverse transcription (RT)- PCR*

Total RNA was extracted from *in vitro* blood-stage cultures infected with *P. falciparum* transgenic strains of interest, or the wild-type 3D7 strain as a control, using a QIAamp RNA Blood Mini Kit (QIAGEN) according to manufacturer's instructions. The extracted RNA was DNase treated using the DNA-*free*™ Kit (Ambion by Life Technologies) according to manufacturer's instructions. Approximately 2 µg of the total RNA was then used to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. The cDNA was tested by PCR amplification of regions specific to the gene of interest using the primers listed in Table 2.7.

| Knock-out construct | Primers for testing cDNA (5'-3') | |
|---|---|---|
| | Forward | Reverse |
| PfDHHC5-KO | CCATTCTTCAATTTGTTGAAG | GGTGTATGGGCCTATAGTACC |
| PfDHHC9-KO | GAATAATTATTTGGCATTTATC | CTCCATCTTCCTTTATGTTTTC |

**Table 2.7: Primers used for the PCR analysis of the cDNA of PfDHHC5-KO and PfDHHC9-KO transgenic clones.**

Quantitative PCR (qPCR) was also performed on the generated cDNA using gene-specific primers, along with gene-specific probes labelled with 6-carboxyfluorescein (6-FAM) on the 5'-end, and a non-fluorescent quencher (MGBNFQ, Applied Biosystems) on the 3'-end. A concentration of 300 nmol/L was used for both primers and probes in a 25 µL reaction (the primers and probes used are listed in Table 2.8). The cycling parameters for the qPCR reaction are as shown below:

| Step | Temperature (°C) | Time (min) |
|---|---|---|
| 1 | 50 | 2 |
| 2 | 95 | 10 |
| 3 | 95 | 0.25 |
| 4 | 60 | 1 |
| 5 | Go to 3, repeat 39 times | |

Each qPCR reaction also included a wild-type 3D7 cDNA control, as well as a standard internal control using the housekeeping gene, seryl-tRNA-synthase (primers and probes used for the seryl-tRNA-synthase housekeeping gene are also shown in Table 2.8). All samples were assayed in triplicate. The qPCR assay described here was kindly performed by Leyla Bustamente, Rayner lab.

| Gene | qPCR primers | | qPCR 6-FAM probe |
|---|---|---|---|
| | Forward | Reverse | |
| PfDHHC5-KO | CCCCCGAATTTACCAGTT | TGCCTTCTTTTGACATATCCTA | CTAAATAGTAAGCAGGTAT |
| PfDHHC9-KO | TCCTACAGTGACATGAATCCTT | AAAACCGCTTTCCAATTATT | TGACTTGGGAATATA |
| Seryl-tRNA-synthase | CCACACAAGGAGAAGATA | GGGGAAAGACAATAGACA | TTAAAGTTTGTTCTCGCTTGAGCCC |

**Table 2.8: Primers and probes used for the qPCR analysis of the cDNA of PfDHHC5-KO and PfDHHC9-KO transgenic clones.**

## 2.4. Human Embryonic kidney 293 (HEK293) *in vitro* cell culture and transfection

### *2.4.1. Human embryonic kidney 293E (HEK293E) cell culture*

Human embryonic kidney 293E (HEK293E) cells were maintained in 'complete' culture media, consisting of Gibco® FreeStyle™ 293 expression media (Life Technologies) supplemented with 1% heat-inactivated FBS under standard humidified conditions (37°C and 5% $CO_2$), essentially as previously described [9], except that the cells were grown in T75 tissue culture flasks (at $1x10^6$ cells/flask) with 25 mL of complete media, instead of in suspension.

### *2.4.2. Transfection of HEK293 cells*

Transient transfection of HEK293 cells with *GeneArt* constructs coding for codon-optimized *P. falciparum* proteins were performed using polyethylenimine (PEI) as previously described [5, 9]. Briefly, cells were split the day before transfection to give a concentration of $1x10^6$ cells per transfection. On the day of transfection, 12.5 µg total DNA was added into 1 mL of unsupplemented FreeStyle media, and mixed with 25 µL of PEI. The transfection mixture was left for 15 minutes at 24°C before being added to the cells, and left overnight under the standard culture conditions described in Section 2.4.1. The transfected cells were collected by centrifugation at 3220xg for 15 minutes and culture supernatants were discarded. For immunofluorescence assays, the cells were split onto poly-l-lysine coverslips (BD Biosciences) in 12-well plates at a concentration of 30000 cells/well in 1 mL of complete culture media the day before transfection. The transfection mix was scaled down appropriately according to the volume of media, and the cells were fixed after overnight transfection as described in Section 2.5.2.

## 2.5. Protein detection and analysis

### *2.5.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting*

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE® Novex® 4-12% Bis-Tris pre-cast gels (Life Technologies), at a constant voltage of 200V for 50 minutes, according to manufacturer's instructions. After electrophoresis was complete, the proteins were then transferred to Immobilon®-P PVDF membranes (Sigma-Aldrich) using the XCell II™ blot module (Life Technologies) and 1X NuPAGE® transfer buffer (Life Technologies) (with 10% methanol), at a constant voltage of 30V for one hour, according to manufacturer's instructions. After the transfer was complete, the PVDF membranes were blocked overnight in 5% milk (Marvel)/PBS. The membranes were probed with primary antibodies, diluted in PBS-0.1% Tween-20 (Sigma-Aldrich) (PBST) or 2% milk/PBS, for 2 hours at 24°C with rotation, followed by 3 washes with PBST. The membranes were then probed with horse radish peroxidase (HRP)-conjugated secondary antibodies (Amersham ECL, GE Healthcare), diluted in PBST or 2% milk/PBS, for 1 hour at room temperature with rotation, followed by another 3 washes with PBST. The presence of the HRP-conjugated secondary antibodies on the membranes was detected using enhanced chemiluminescence (Amersham ECL, GE Healthcare). All primary and secondary antibodies used are listed in Table 2.9 with the appropriate dilutions.

| Antibodies | | Source | Dilution | |
|---|---|---|---|---|
| | | | IFA | Western blot |
| Primary | Anti-HA-tag mouse monoclonal (6E2) | Cell Signaling Technology | 1:200 | 1:400 |
| | Anti-HA-tag rabbit monoclonal (C29F4) | | 1:200 | 1:400 |
| | Anti-HA-tag rabbit polyclonal | Life Technologies | - | 1:400 |
| | Anti-*P. falciparum* ERD2 rabbit polyclonal (MRA-1) | MR4, ATCC | 1:2000 | - |
| | Anti-*P. falciparum* GRP (BiP) rat polyclonal | MR4, ATCC | 1:2000 | - |
| | Anti-*P. falciparum* GAP45 rabbit | Matt Jones | 1:1000 | - |
| | Anti-*P. falciparum* MSP1 mouse monoclonal (MRA-94) | MR4, ATCC | 1:1000 | - |
| | Anti-*P. falciparum* RAP1 mouse monoclonal (MRA-79) | MR4, ATCC | 1:1000 | - |
| | Anti-*myc* mouse monoclonal (9E10) | Life Technologies | 1:1000 | 1:500-1:1000 |
| | Anti-c-myc rat monoclonal (JAC6) | Abcam | 1:1000 | 1:500-1:1000 |
| | Anti-FLAG® mouse monoclonal (M2) | Sigma Aldrich | 1:1000 | 1:1000 |
| | Anti-FLAG® rabbit polyclonal | Sigma Aldrich | 1:1000 | 1:1000 |
| | Anti-calnexin rabbit polyclonal | Abcam | 1:1000 | - |
| | Anti-pan-cadherin rabbit polyclonal | Abcam | 1:1000 | - |
| Secondary | Alexa Fluor® 488 Goat Anti-Mouse/Rabbit/Rat IgG (H+L) | Life Technologies | 1:1000 | - |
| | Alexa Fluor® 555 Goat Anti-Mouse/Rabbit/Rat IgG (H+L) | Life Technologies | 1:500-1:1000 | - |
| | Amersham ECL™ HRP-linked Anti-Mouse/Rabbit/Rat IgG | GE Healthcare | - | 1:4000 |

**Table 2.9: All primary and secondary antibodies used in this work along with their appropriate working dilutions.**

*2.5.2. Immunofluorescence Assay*

***In suspension:*** The pellet from 600 µL of *in vitro* blood-stage culture infected with *P. falciparum* transgenic strains of interest was collected by centrifugation at 450xg for 1 minute, and fixed in 4% formaldehyde/0.01% gluteraldehyde/PBS at for one hour at 24°C with rotation. The fixed pellet was then permeabilised with 0.1% Triton X-100 for 10 minutes at 24°C with rotation, followed by blocking in 3% bovine serum albumin (BSA) (Sigma-Aldrich)/PBS for one hour at 24°C with rotation. Immunodetection was performed by incubating the fixed cell pellets with primary antibodies, diluted in 1% BSA/PBS (all primary antibodies used and the corresponding dilutions are shown in Table 2.9), for 2 hours at 24°C with rotation. After 3 washes in 1% BSA/PBS, this was followed by incubation with secondary antibodies, diluted in 1% BSA/PBS (all secondary antibodies used and the corresponding dilutions are shown in Table 2.9), for 1 hour at 24°C with rotation. During the secondary antibody incubation, the nuclear DNA was also stained with DAPI at a concentration of 1:4000. After a final 3 washes, the pellet was resuspended in 250 µL FBS and 7 µL of the resuspended pellet was spread out on a glass slide and allowed to dry. This was then mounted in Prolong anti-fade mounting reagent (Life Technologies).

***On poly-l-lysine coverslips:*** Poly-l-lysine coverslips (BD Biosciences) were placed into the wells of 12-well plates and cells were either grown on the poly-l-lysine coverslips (as described in Section 2.4.2), or dropped onto the coverslips (as in Section 2.2.7) and left for 15 minutes to attach. The cells were then fixed in 500 µL of 4% formaldehyde/0.01% gluteraldehyde/PBS for 15 minutes at 24°C. The permeabilisation, blocking, and primary and secondary antibody incubation steps were performed as described above, except that all incubations were done in the wells of the 12-well plate without rotation. After the final washes, the coverslips were removed from the 12-well plate and mounted onto glass slides using Prolong anti-fade mounting reagent (Life Technologies).

For both methods of immunofluorescence assay described above (in suspension and on coverslips), confocal images were acquired using a Zeiss LSM 510 Laser Scanning confocal microscope.

*2.5.3. Immunoprecipitation of c-Myc-tagged proteins*

HEK293 cells were either co-transfected with both the FLAG-tagged PfDHHC protein of interest and a c-Myc-tagged potential substrate, or co-transfected with the c-Myc-tagged potential substrate and the empty CD4 vector control, as described in Section 2.4.2. After 24 hours of transfection at 37°C under standard culture conditions, transfected cells were collected by centrifugation at 3220xg for 10 minutes. The cell pellets were lysed in 500 µL of IP buffer (1% Triton X-100, 50 mM Tris-Cl pH 7.4, 150

mM NaCl, 5 mM EDTA, protease inhibitor cocktail (Roche)) and incubated for 30 minutes at 37°C with shaking. Cell debris was removed by centrifugation at 20238xg for 5 minutes, and the resulting supernatants were pre-cleared by incubating with 15 µL of Protein G Sepharose® (Sigma-Aldrich) per sample for 1 hour at 4°C with rotation. The Protein G Sepharose resin was collected by centrifugation at 20238xg for 1 minute at 4°C. The resulting supernatants were then incubated with 2 µg of mouse α-c-Myc antibody (Life Technologies) per sample and incubated overnight at 4°C with rotation. This was followed by incubation with 30 µL of Protein G Sepharose per sample for 2 hours at 4°C with rotation. The Protein G Sepharose resin was collected by centrifugation at 20238xg for 5 minutes at 4°C and subjected to 3 washes with IP buffer. The immunoprecipitated proteins were then eluted from the Protein G Sepharose beads by incubating with 60 µL of 2% SDS/50 mM Tris-Cl pH 7.4/5 mM EDTA for 5 minutes at 95°C with shaking. For SDS-PAGE and Western blot analysis, 10 µL of each eluate was diluted in 2X Laemmli sample buffer (4% SDS, 120 mM Tris-Cl pH 6.8, 20% glycerol, 0.02% bromophenol blue) with 10% β-mercaptoethanol (Sigma-Aldrich) and analysed as described in Section 2.5.1.

## 2.6. Palmitome purification in *P. falciparum* parasites and HEK293 cells

### *2.6.1. Purification of palmitoylated peptides in P. falciparum schizonts using Acyl-biotinyl exchange (ABE) –Trial 1*

Parasites were extracted from *in vitro* blood-stage cultures infected with *P. falciparum* strain 3D7 by saponin-lysis (described in 2.2.5). The saponin-extracted parasite pellet was first washed in ice cold ABE lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA) and resuspended at a concentration of $1x10^9$ parasites/mL in ice-cold ABE lysis buffer containing 1.7% Triton X-100, 10 mM N-ethylmaleimide (NEM) (Sigma-Aldrich), Protease inhibitor cocktail (Sigma-Aldrich) and Phosphatase inhibitor cocktail 2 (Calbiochem). The lysates were incubated for one hour at 4°C with rotation, followed by precipitation of proteins from the lysates by chloroform-methanol (C/M) precipitation. Precipitated proteins were resuspended in 4% SDS/50 mM Tris-Cl pH 7.4/5 mM EDTA/10 mM NEM and incubated at 37°C with shaking until fully solubilised. The solubilised proteins were diluted in ABE lysis buffer containing 1 mM NEM and 0.2% Triton X-100, and incubated overnight at 4°C with rotation. The samples were then subjected to 3 sequential C/M precipitations to ensure all NEM was removed. After the final C/M precipitation, the samples were split into two equal portions. One portion was treated with hydroxylamine by resuspension in (+)hydroxylamine buffer (0.7 M hydroxylamine (Sigma-Aldrich) pH 7.4, 1 mM EZ-link HPDP-biotin (Thermo Scientific),

0.2% Triton X-100), and the second portion was mock treated by resuspension in (–)hydroxylamine buffer (50 mM Tris-Cl pH 7.4, 1 mM EZ-link HPDP-biotin, 0.2% Triton X-100). The (+) and (-) hydroxylamine treatments were incubated for one hour at 24°C with rotation, followed by C/M precipitation. The precipitated samples were then further treated with HPDP-biotin by resuspension in HPDP-biotin buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5mM EDTA, 0.2 mM EZ-link HPDP-biotin, 0.2% Triton X-100) for 2 hours at 24°C with rotation. The HPDP-biotin buffer was removed by 3 sequential C/M precipitations and the precipitated proteins were resuspended in 2% SDS/50 mM Tris-Cl pH 7.4/5 mM EDTA.

The samples were then diluted in ABE lysis buffer containing 0.2% Triton X-100, at a volume that resulted in the dilution of the SDS concentration in the samples to 0.1%. Streptavidin-agarose resin (Thermo Scientific), at a bed volume of 200 µL, was added into each sample and the samples were incubated for 2 hours at 24°C with rotation. The streptavidin-agarose resin was precipitated by centrifugation at 3200xg for 2 minutes and washed once with ABE lysis buffer for 10 minutes. This was followed by a further 3 washes with 2 M urea (Sigma-Aldrich)/100 mM ammonium bicarbonate (Sigma-Aldrich), at 10 minutes per wash. The resin was then treated with 20 µg of Trypsin Gold (Promega) in 1 mL total volume of 1 M urea/50 mM ammonium bicarbonate. Trypsin digestion was allowed to proceed for 2 hours at 37°C with shaking. The resin was then loaded into microcentrifuge spin columns (Thermo Scientific) and washed 3 times with 2 M urea/100 mM ammonium bicarbonate, using syringes. Peptides were then eluted from the resin by incubating with 50 µL of 10 mM Tris(2-carboxyethyl)phosphine(TCEP) for 10 minutes at 37°C with shaking. This was repeated with a further 50 µL of TCEP and both elutions were pooled. The purification methods described above were performed in collaboration with Mark Collins, Choudhary lab.

For quantitative analysis of the eluted peptides, samples were desalted and subjected to stable isotope dimethyl labelling as previously described [10]. Briefly, (-)hydroxylamine samples were labelled with ‘medium’ deuterated formaldehyde (D2) and sodium cyanoborohydride, and the (+)hydroxylamine samples were labelled with ‘heavy’ $^{13}$C-deuterated formaldehyde (13C-D2) and sodium cyanoborodeuteride. Labelled peptides were pooled and acidified using 10% formic acid for analysis by LC-MS/MS with an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). The stable isotope dimethyl labelling and LC-MS/MS analysis was kindly performed by Mark Collins, Choudhary lab.

*2.6.2. Purification of palmitoylated peptides in P. falciparum schizonts using Acyl-biotinyl exchange (ABE) – Trial 2, 3A, 3B and 4*

Parasites were extracted from *in vitro* blood-stage cultures infected with *P. falciparum* strain 3D7 by saponin-lysis (described in 2.2.5). The saponin-extracted parasite pellet was lysed in 1 mL of extraction buffer (4% SDS, 0.1 M Tris-Cl pH 8.0, 2 µg/µL Aprotinin/Leupeptin, 0.5 mM PMSF, 20 µM ZnCl, 5 mM EDTA and 25 mM TCEP) and homogenised 25 times. The homogenised lysate was then heated for 10 minutes at 70°C and passed through a fine gauge needle 10 times to sheer the DNA. The lysates were subjected to centrifugation at 20238xg for 5 minutes and the supernatants transferred to a new tube. The remaining pellet was then further lysed in 400 µL 8 M urea/100 mM Tris-Cl pH 8.0, and vortexed for 2 minutes. This urea-treated lysate was subjected to centrifugation at 20238xg for 5 minutes. The resultant supernatant was pooled with the first supernatant, and a 10 µL aliquot of the pooled lysates was kept aside for measurement of protein concentration. Protein concentration was measured using the QuantiPro BCA Assay Kit (Sigma-Aldrich) according to manufacturer's instructions. Iodoacetamide (IAA) (Sigma-Aldrich) was added to the lysate at a final concentration of 50 mM. Urea was also added to a final concentration of 8 M. The samples were incubated for 2 hours at 24°C in the dark with rotation. Cysteine, at a final concentration of 50 mM, was then added to the sample and incubated for 30 minutes at 24°C with rotation, to quench the remaining IAA.

The lysate was then split into two equal portions and transferred into two pre-washed (wash buffer: 8 M urea/100 mM Tris-Cl pH 8.0) Amicon Ultra-15 Centrifugal Filter Units (30 kDa molecular weight cut-off) (Millipore). The samples were washed 4 times with 2 mL 8 M urea/100 mM Tris-Cl pH 8.0 buffer. To one sample – the (+)hydroxylamine sample- the following reagents were added to the upper chamber of the filter units: 210 µl 100 mM Tris-Cl pH 7.4, 20 µL 50 mM EZ-link HPDP-biotin and 350 µL 2 M hydroxylamine pH 7.4. To the second sample – the (-)hydroxylamine sample- the following reagents were added to the upper chamber of the filter units: 560 µL 100 mM Tris-Cl pH 7.4 and 20 µL 50 mM EZ-link HPDP-biotin. Samples were fully resuspended and incubated for one hour at 24°C with shaking. The ABE reagents were removed by centrifugation at 3220xg for 20 minutes, followed by 4 washes with 2 mL 8M urea/100 mM ammonium bicarbonate buffer and a final wash with 1 mL 100 mM ammonium bicarbonate buffer. The samples were made up to 2 mL with 100 mM ammonium bicarbonate buffer and the appropriate amount of Trypsin Gold (Promega) was added to each sample at an enzyme:substrate ratio of 1:50. The samples were digested for 4 hours at 37°C with shaking. The digested peptides were collected by centrifugation at 3220xg for 15 minutes. The upper chambers of the spin columns were then washed with 2 mL wash buffer (100

mM Tris-Cl pH 7.4, 300 mM NaCl, 10mM EDTA pH 7.4, 0.2% SDS, 0.4% Triton X-100), and the wash buffer pooled with the previously collected peptides.

Streptavidin-agarose resin (at a bed volume of 200 µL per sample) was washed with LB buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 7.4, 0.1% SDS, 0.2% Triton X-100), and added to the peptide samples. The samples were incubated for one hour at 24°C with rotation. The resins were then washed twice with 10 mL of LB buffer (at 10 minutes per wash), and washed a further two times with 10 mL of 2 M urea/100 mM ammonium bicarbonate buffer (at 5 minutes per wash). The resins were loaded into microcentrifuge spin columns and washed a further two times with 10 mL of 2 M urea/100 mM Tris-Cl pH 7.4 buffer, followed by one wash with 2 mL water, using syringes. Peptides were then eluted from the resin by incubating with 50 µL 10 mM TCEP for 10 minutes at 37°C with shaking. This was repeated with another 50 µl of 10 mM TCEP, and both elutions were pooled. The eluted peptides were either subjected to stable isotope dimethyl labelling followed by acidification with 10% formic acid as described above (Section 2.6.1), or acidified with 10% formic acid without labelling (for label-free analysis). The labelled and label-free peptides were then analysed by LC-MS/MS with an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). The purification methods described above were performed in collaboration with Mark Collins, Choudhary lab. Stable isotope dimethyl labelling and LC-MS/MS analysis was kindly performed by Mark Collins, Choudhary lab.

### *2.6.3. Purification of palmitoylated proteins in HEK293 cells by metabolic labelling and click chemistry*

HEK293 cells –either co-transfected with both the FLAG-tagged PfDHHC protein of interest and a c-Myc-tagged potential substrate, or co-transfected with the c-Myc-tagged potential substrate and the empty CD4 vector control- were treated with 25 µM of the palmitic acid analogue, 17-octadecynoic acid (17-ODYA) (Cayman Chemical), or mock-treated with an equal volume of DMSO (Sigma-Aldrich), 24 hours after transfection. The 17-ODYA treatment was allowed to continue for a further 6 hours at 37°C under the standard culture conditions described in Section 2.4.1. The transfected and 17ODYA-labelled cells were collected by centrifugation at 3220xg for 15 minutes and the supernatants were removed. The resulting cell pellets were each lysed in 500 µL of buffer containing 1% Triton X-100/50 mM Tris-Cl pH 7.4/ 150 mM NaCl/EDTA-free protease inhibitor cocktail (Roche), and incubated for 30 minutes at 37°C with shaking. The lysates were subjected to centrifugation at 20238xg for 5 minutes and the supernatants were C/M precipitated and then resuspended in 2% SDS/PBS. Protein concentrations were measured for all conditions using the QuantiPro BCA assay kit (Sigma-Aldrich) according to manufacturer's instructions. The click chemistry reaction was set up with approximately 2 mg of protein for each of the conditions using the following chemicals: 100 µM biotin-azide

(Invitrogen), 1 mM TCEP (Sigma-Aldrich), 100 µM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, Sigma-Aldrich) dissolved in DMSO/*tert*-butanol (20%/80%), and 1 mM $CuSO_4$ (Sigma-Aldrich). The reaction was made up to a total volume of 500 µL with PBS and allowed to proceed for 1.5 hours at 23°C. C/M precipitation was then performed to completely remove the reactants of the click chemistry reaction. The precipitated proteins were resuspended in 2% SDS/PBS and diluted 10X with 0.2% Triton X-100/PBS. Pre-washed streptavidin-agarose resin (wash buffer: 0.2% Triton X-100/PBS) was added to the samples, at 50 µL bed volume of streptavidin-agarose per sample, and incubated for 2 hours at 24°C with rotation. The streptavidin-agarose resins were washed 4 times with 0.2% Triton X-100/PBS. Proteins were eluted from the resins by adding 100 µL of 2% SDS/50 mM Tris-Cl pH 7.4, 5 mM EDTA pH 7.4 and incubating for 5 minutes at 95°C with shaking. For SDS-PAGE and Western blot analysis, 12 µL of each eluate was diluted in 2X Laemmli sample buffer (4% SDS, 120 mM Tris-Cl pH 6.8, 20% glycerol, 0.02% bromophenol blue) with 10% β-mercaptoethanol (Sigma-Aldrich) and analysed as described in Section 2.5.1.

## References:


1. Abdi, A., et al., *SAM domain-dependent activity of PfTKL3, an essential tyrosine kinase-like kinase of the human malaria parasite Plasmodium falciparum.* Cellular and Molecular Life Sciences, 2010. **67**(19): p. 3355-3369.
2. Maier, A.G., et al., *Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination.* Mol Biochem Parasitol, 2006. **150**(1): p. 118-121.
3. Pfander, C., et al., *A scalable pipeline for highly effective genetic modification of a malaria parasite.* Nat Methods, 2011. **8**(12): p. 1078-1082.
4. Janse, C.J., J. Ramesar, and A.P. Waters, *High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei.* Nat Protoc, 2006. **1**(1): p. 346-356.
5. Durocher, Y., S. Perret, and A. Kamen, *High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells.* Nucleic Acids Res, 2002. **30**(2): p. E9.
6. Trager, W. and J.B. Jensen, *Human malaria parasites in continuous culture.* Science, 1976. **193**(4254): p. 673-5.
7. Crabb, B.S., et al., *Transfection of the human malaria parasite Plasmodium falciparum.* Methods Mol Biol, 2004. **270**: p. 263-76.
8. Theron, M., et al., *An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by Plasmodium falciparum parasites.* Cytometry Part A, 2010. **77A**(11): p. 1067-1074.
9. Bushell, K.M., et al., *Large-scale screening for novel low-affinity extracellular protein interactions.* Genome Research, 2008. **18**(4): p. 622-630.
10. Boersema, P.J., et al., *Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics.* Nat Protoc, 2009. **4**(4): p. 484-494.