Role of *Plasmodium falciparum* transporters in drug resistance:

- Characterization of the putative organic cation transporter PFE0825w in *Xenopus laevis* oocytes.

- Analysis of the role of phosphorylation in the drug-resistance-mediating function of the chloroquine resistance transporter PfCRT.

Dissertation

Sonia Moliner Cubel

Referees: **Prof. Dr. Michael Lanzer**
Prof. Dr. Christine Clayton

Dissertation submitted to the Combined Faculties for the Natural Sciences and for Mathematics of the Ruperto-Carola University of Heidelberg, Germany for the degree of Doctor of Natural Sciences

presented by
Sonia Moliner Cubel
born in Barcelona, Spain

Date of oral examination: ……

Role of *Plasmodium falciparum* transporters in drug resistance:

- Characterization of the putative organic cation transporter PFE0825w in *Xenopus laevis* oocytes.

- Analysis of the role of phosphorylation in the drug-resistance-mediating function of the chloroquine resistance transporter PfCRT.

Referees: **Prof. Dr. Michael Lanzer**
Prof. Dr. Christine Clayton

Ich erkläre hiermit, dass ich die vorliegende Doktorarbeit selbstständig unter Anleitung verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Ich erkläre hiermit, dass ich an keiner anderen Stelle ein Prüfungsverfahren beantragt bzw. die Dissertation in dieser oder anderer Form bereits anderweitig als Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt habe.

Die vorliegende Arbeit wurde am Department für Infektiologie, Abteilung Parasitologie des Universitätsklinikum Heidelberg in der Zeit von Oktober 2011 bis April 2016 unter der Leitung von Prof. Dr. Michael Lanzer ausgeführt.

……. .....

Datum Sonia Moliner Cubel

Table of Contents

Acknowledgements

Thanks to Prof. Dr. Michael Lanzer for giving me the opportunity to do my PhD work in his group. Thanks for all the support and guidance over these years.

Thanks to my second supervisor Prof. Dr. Henri Vial, to Prof. Dr. Christine Clayton and to Dr. Gabrielle Planelles for all the recommendations, suggestions and advices during all TAC meetings. Thanks to Dr. Dirk Grimm for participating in my defense committee. Thanks also to Dr. Cecilia Sanchez for introducing me to the different lab techniques, for discussing with me about the results always when I needed, for her ideas and her trust in me.

Thanks to all the parasitology department of Heidelberg University for the great discussions on the Friday seminars and for creating such a thrilling scientific atmosphere around me.

Thanks to all my lab mates for their help during all these years. Thanks to Marina, Sophia, Katharina, Hani, Caro, Nicole, Ines, Carine, Tina, Mailin, Marvin, Eike, Maëlle, Monika, Isa, Britta and Gesa for being always there when I needed them, for the interesting scientific and non-scientific discussions and for creating such a nice work environment. Thanks especially to Sebastiano and Anurag for introducing me to the oocyte techniques, to Martin for his help with statistics and to Nick for correcting the English of all my reports. Thanks to all the students that I had the pleasure to supervise. Thanks especially to Maike and Clemens for their interest, their motivation and for teaching me as much as I taught them.

Thanks to EVIMalaR for creating such a wonderful PhD program. Thanks to Jaishree, Sonal, Jenny, Gabriella, Pablo, George, Harshal, Ahmed and Sam for the great time we spend together during the core course and all meetings and conferences. Thanks to Kartik for being my best friend here in Heidelberg and for his unconditional help. Thanks to Mariana for sharing with me her passion for science, for being always there to listen to my existential questions and for making me feeling I was not alone during this journey.

Thanks to Miriam, Alessia, Elisa and Sandra for all their help with the administrative paper work. Thanks for receiving me always with a smile and willing to help me with any sort of problem or question.

Thanks to all the persons that encouraged me to do this PhD. Thanks especially to Alfonso and María for making me grow as a scientist and for their trust and motivation.

Thanks to all my friends that morally support me during this time. Thanks to everybody that came to visit me and that despite the distance I still feel really close. Thanks to Àlex for helping me to prepare the interviews. Thanks to Eva for being my first visit and the first one I could share my first impressions with. Thanks to Miriam for sharing her passion for traveling and for the long phone conversations that made me feel we were not so far away. Thanks to Mar, Elba and Anna for being the best friends that one could ever have. Thanks to Helena, Laia, Lara, Aurora and Eli for sharing with me the important moments of their lives. Thanks to Delia, Noe, Rubén, Núria, Marta, Bea and Lidia for making me feel that Madrid is my second home. Thanks to Laura, Sandra, Javi and Victor for making me laugh so much and for sharing so many funny moments with me. Thanks to Sarah and Medhi for making me part of their lives.

Thanks to Beate and Wolfgang for accepting me as part of their family and for their support in everyday things.

Thanks to Stefan for all his support during these years, because without him maybe I would not be writing this thesis. Thanks for being always comprehensive when I worked too much. Thanks for all the times that you drove me to the lab and for all the times that you had to wait for me. Thanks for seeing always the best of me and for motivating me when I was down. Thanks for making me happy, for sharing your life with me and for all your love.

Gracias a toda mi familia, no se puede tener una mejor. Gracias a mi hermana Ana y mi cuñado Jordi por su apoyo, sus ánimos y su cariño. Gracias a mis sobrinos Emma y Asier por hacerme feliz sólo con sonreír. Y la mayor de las gracias a mis padres Javier y Carmen. Gracias por darme la educación que vosotros no pudisteis tener. Gracias por todos los sacrificios que habéis hecho por mí y por anteponer mis necesidades a las vuestras. Gracias por apoyarme en mis decisiones. Gracias por confiar siempre en mí, por vuestro amor y vuestros consejos.

Summary

Although the fight against malaria has achieved a remarkable progress during the last 15 years, there were still 214 million new cases and 438000 estimated deaths caused by malaria worldwide in 2015. Transporters play a crucial role in *Plasmodium* biology but they can also be considered as double edge swords: on the one hand, they are potential new antimalarial drug targets but on the other hand, they are the main players in the development of drug resistance. PFE0825w is a putative organic cation transporter that has been proposed as the target of the candidate antimalarial drug albitiazolium and the locus where it is localized has been linked to chloroquine transport. In this study, different PFE0825w isoforms were identified and studied using the *X. laevis* oocyte system. Two of the isoforms were expressed at the oocyte oolemma but no significant transport of putative organic cation substrates was detected, restricting further characterization of this transporter. A better characterized transporter that plays a significant role in resistance against chloroquine (CQ) and quinine (QN) is the chloroquine resistance transporter PfCRT. It is known that this transporter has at least three different phosphorylation sites and that the phosphorylation of one of these sites -T^{416}- is essential for the correct trafficking of PfCRT to the food vacuolar membrane. In this study the role of phosphorylation in the drug-resistance-mediating function of PfCRT was investigated. CQ-resistant parasites treated with the kinase inhibitor ML-7 accumulated more CQ than untreated parasites and showed CQ and QN IC_{50} values comparable to those of sensitive strains. Along the same line, the mutagenesis of the phosphorylation site S^{33} to alanine in PfCRT led to reduced CQ and QN IC_{50} values although no increase in drug accumulation was observed. Furthermore, $PfCRT^{S33A}$ conferred a fitness advantage to the parasites in the absence of CQ and a fitness cost in the presence of the drug. Two protein kinases were analyzed regarding their roles in PfCRT phosphorylation, PfCK2 and PF11_0488, the latter being identified in a Y2H assay. The downregulation of PfCK2 did not have an effect on CQ accumulation, but the overexpression of the C-terminal part of PF11_0488 resulted in reduced levels of CQ accumulation. However, the same fragment did not show any catalytic activity when recombinantly expressed and used in *in vitro* phosphorylation assays. Downregulation of this kinase was not achievable, most likely due to its essential function. Altogether, these results point to the fact that the parasite susceptibility towards CQ and QN is regulated by phosphorylation, although the exact molecular mechanism needs to be further examined.

Zusammenfassung

Obwohl der Kampf gegen Malaria in den letzten 15 Jahren bemerkenswerte Fortschritte gemacht hat, gab es im Jahr 2015 noch 214 Millionen Erkrankungsfälle und schätzungsweise 438.000 durch Malaria verursachte Todesfälle weltweit. Transport-Proteine spielen eine entscheidende Rolle in der Biologie von Plasmodium, aber sie können auch als eine zweischneidige Klinge angesehen werden: auf der einen Seite sind sie potenziell neue Ansatzpunkte für Anti-Malaria-Wirkstoffe, auf der anderen Seite die wichtigsten Akteure in der Entwicklung von Resistenzen. PFE0825w ist ein putativer Transporter für organische Kationen, der als Ansatzpunkt des potentiellen Antimalariamittels Albitiazolium vorgeschlagen wurde und der Ort, in dem es lokalisiert ist, wurde mit Chloroquin-Transport in Verbindung gebracht. In der vorliegenden Arbeit wurden verschiedene Isoformen von PFE0825w identifiziert und mit Hilfe des X. laevis Oozyten Systems untersucht. Zwei dieser Isoformen wurden auf dem Oolemma der Oozyte exprimiert, jedoch konnte kein signifikanter Transport von organischen Kationen nachgewiesen werden, was eine weitere Charakterisierung dieses Transporters eingrenzt. Ein besser charakterisierter Transporter, der eine bedeutende Rolle für die Resistenz gegen Chloroquin (CQ) und Chinin (QN) spielt, ist der Chloroquin Resistenz Transporter PfCRT. Es ist bekannt, dass dieser Transporter mindestens drei unterschiedliche Phosphorylierungsstellen besitzt und dass die Phosphorylierung an einer dieser Stellen - T^{416} - wesentlich ist, um korrekt an die Nahrungsvakuolen-Membran dirigiert zu werden. In dieser Studie wurde die Rolle der Phosphorylierung hinsichtlich der Medikamenten-Resistenz vermittelnden Funktion von PfCRT untersucht. CQ resistente Parasiten, welche mit dem Kinase-Inhibitor ML-7 behandelt wurden, akkumulierten mehr CQ als unbehandelte Parasiten und zeigten mit sensitiven Stämmen vergleichbare IC_{50}-Werte für CQ und QN. Genauso führte die Mutation der Phosphorylierungsstelle S^{33} zu Alanine in PfCRT zu verringerten IC_{50}-Werten für CQ und QN, obgleich keine Zunahme der Medikamenten-Akkumulation beobachtet wurde. Darüber hinaus vermittelte $PfCRT^{S33A}$ für die Parasiten einen Selektionsvorteil in Abwesenheit von CQ und einen Nachteil in Anwesenheit des Wirkstoffs. Es wurden zwei Proteinkinasen bezüglich ihrer Bedeutung für die Phosphorylierung von PfCRT untersucht, PfCK2 und PF11_0488, wobei letztgenannte in einem Y2H Versuchsansatz identifiziert wurde. Das Herunterregulieren der PfCK2 hatte keine Auswirkung auf die CQ-Akkumulation, die Überexpression des C-terminalen Teils von PF11_0488 jedoch führte zu einer verringerten

Anhäufung von CQ. Allerdings zeigte das gleiche Fragment keine katalytische Aktivität, wenn es rekombinant exprimiert und in in vitro Phosphorylierungs-Versuchsansätzen verwendet wurde. Eine Herunterregulation dieser Kinase konnte nicht erreicht werden, wahrscheinlich wegen ihrer essentiellen Funktion. Insgesamt deuten diese Ergebnisse darauf hin, dass die Anfälligkeit des Parasiten gegen CQ und QN durch Phosphorylierung reguliert wird, der genaue molekulare Mechanismus bedarf jedoch weiterer Untersuchungen.

Abbreviations

A	Adenine or alanine
ACT	Artemisinin combination therapy
ADP	Adenosine diphosphate
AMA1	Apical membrane antigen 1
AMP	Adenosine monophosphate
AmpR	Ampicillin resistance gene
AP2-G	Activator protein 2 - gametogenesis
APS	Ammonium persulphate
AQP	Aquaporin
AS	Alternative splicing
ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
ATP4	ATPase 4
ATP6	ATPase 6
BIP	Binding protein
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine or cysteine
C-terminal	Carboxy terminal
C3	3 carbon
CAD	Conditional aggregation domain
Cas	CRISPR associated
CBS	Calmodulin binding site
cDNA	Complementary DNA
CDPK	Calcium dependent protein kinase
CHA	Calcium hydrogen antiporter
CHT	Choline high-affinity transporter
CIP	Calf intestinal alkaline phosphatase
CK	Casein kinase
CLAG	Cytoadherence linked antigen
CNRS	*Centre national de la recherche scientifique*
CQ	Chloroquine
CQR	Chloroquine resistance

CQS	Chloroquine sensitive
CRISPR	Interspaced short palindromic repeats
CRT	Chloroquine resistance transporter
CSP	Circumsporozoite protein
CuP-ATPase	Copper P-ATPase
CysRS	Cysteinyl tRNA synthetase
D	Aspartic acid
Da	Dalton
DD	Destabilization domain
ddH_2O	Double distilled water
DDD	DHFR degradation domain
DHFR	Dihydrofolate reductase
DHHC	Asparagine histidine histidine cysteine protein
DHP	Heme detoxification protein
DHPS	Dihydropteroate synthase
DMSO	Dimethylsulfoxide
DMT	Drug metabolite transporter
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DO	Dropout
DOC	Sodium deoxycholate
DSBR	Double-strand break repair
dsDNA	Double stranded DNA
DT	Dihydrofolate reductase-thymidylate synthase
DTT	Dithiothreitol
DV	Digestive vacuole
DXP	1-deoxy-d-xylulose 5-phosphate
E	Glutamic acid
E.	*Escherichia*
EBL	Erythrocyte binding like
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylenglycoltetraacetic acid
EJ	End joining

eIF2α	Eukaryotic initiation factor 2 α
EMP1	Erythrocyte membrane protein 1
ER	Endoplasmic reticulum
et al.	*Et alia* (and others)
EtBr	Ethidium bromide
F	Phenylalanine or filial
F-type	F_0F_1 type
FBP	Fibronectin binding protein
Fig.	Figure
FIKK	Phenylalanine, isoleucine, lysine, lysine motif
FKBP	FK506 binding protein
FNP	Formate-nitrite transporter
for	Forward
FP	Ferriprotoporphyrin IX
frt	FLP recombinase target
FV	Food vacuole
G	Glycine or guanine
g	Gram(s) or gravitational force
G6PD	Glucose 6-phosphate 1-dehydrogenase
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GAP45	Glideosome associated protein 45
GFP	Green fluorescence protein
GlcN6P	Glucosamine-6-phosphate
GlmS	Glucosamine-6-phosphate synthase
GLUT1	Glucose transporter 1
GP	Glycerophosphate
GST	Glutathione S-transferase
h	Hour or human
H	Histidine
HA	Hemagglutinin
HD	High definition
HeLa	Henrietta Lacks
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
His	Histidine tag
HR	Homologous recombination

HT	Hexose transporter
I	Isoleucine
IC_{50}	Half maximal inhibitory concentration
$InsP_3R$-II	Inositol 1,4,5-trisphosphate receptor type II
iRBC	Infected red blood cell
iTPT	Inner membrane triosephosphate transporter
K	Lysine
K1\Kch1	Potassium channel 1
K2\Kch2	Potassium channel 2
Kb	Kilobase
KO	Knock out
l	Liter
L	Leucine
LB	Luria-Bertani
loxP	Locus of crossover in P1
m	Milli- or meter
M	Molar or methionine
MAEBL	Merozoite apical erythrocyte binding ligand
MAHRP2	Membrane-associated histidine-rich protein-2
MAP2	Mitogen-activated protein kinase 2
MAPKAP	(Mitogen activated protein kinase)-activated protein
MDR1	Multidrug resistance protein 1
MES	2-(N-morpholino)ethanesulfonic acid
MFS	Major facilitator superfamily
min	Minute(s)
MLCK	Myosin light chain kinase
MMJE	Microhomology-mediated end joining
MMLV	Moloney murine leukemia virus
MOPS	3-(N-morpholino) propanesulfonic acid
MPP	1-methyl-4-phenylpyridinium
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MSK1	Mitogen and stress activated protein kinase 1
MSP1	Merozoite surface protein 1
MTIP	Myosin A tail domain interacting protein

n	Nano
N	Asparagine
NEB	New England Biolabs
NHE1	Sodium hydrogen exchanger 1
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
nm	Nanometer(s)
NP40	Nonyl phenoxypolyethoxylethanol 40
NPPs	New permeation pathways
NSC	Negative selection cassette
NT1	Nucleoside transporter 1
OCT	Organic cation transporter
OD	Optical density
OR2	Oocyte ringer solution
oTPT	Outer membrane triosephosphate transporter
P	Proline or phosphorylation
P.	*Plasmodium*
P-type	Phosphorylation type
PAGE	Polyacrylamide gel electrophoresis
Pat	Putative anion transporter
Pb	*Plasmodium berghei*
PBK	PDZ binding kinase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline supplemented with Tween-20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEXEL	Plasmodium export element
Pf	*Plasmodium falciparum*
pH	Power of hydrogen
PI	Phosphatidylinositol or protease inhibitors
PI3K	Phosphatidylinositol 3 kinase
PiT	Inorganic phosphate transporter
PKA	Protein kinase A
PKG	Protein kinase G
PMCA	Plasma membrane calcium pump

pmol	Picomole(s)
POD	Peroxidase
PSC	Positive selection cassette
PTEX	*Plasmodium* translocon of exported proteins
PTMs	Post-translational modifications
PV	Parasitophorous vacuole
PVDF	Polyvinylidene difluoride
PVM	Parasitophorous vacuolar membrane
Q	Glutamine
QN	Quinine
R	Arginine
RAMA	Rhoptry associated membrane antigen
RBC	Red blood cell
rev	Reverse
Rh	Reticulocyte binding like
RIFIN	Repetitive interspersed family
RIPA	Radioimmunoprecipitation assay b
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROCK-II	Rho-associated protein kinase II
RON2	Rhoptry neck protein 2
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
S	Serine
S.	*Saccharomyces* or *Streptococcus*
S6K1	Ribosomal protein S6 kinase beta-1
SAP	Shrimp alkaline phosphatase
SB	Super broth
SD	Synthetic defined
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERA5	Serine repeat antigen 5
sgRNA	Single guide RNA
SOB	Super optimal broth

SOC	Super optimal broth with catabolite repression
SRC	Sarcoma
SSA	Single strand annealing
SSC	Saline sodium chloride
STEVOR	Subtelomeric variant open reading frame
SUB1	Subtilisin like protease 1
T	Thymine or threonine
T4	Bacteriophage T4
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TB	Tris-Borate
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEA	Tetraethylamonium
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TetO	Tetracycline operator
TetR	Tetracycline repressor
TM	Transmembrane
TRAP	Thrombospondin related adhesive protein
Tris	Trisaminomethane
TRSP	Thrombospondin related sporozoite protein
U	Unit(s)
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet
V	Valine or volt
V-PPases	Vacuolar type H^+ pyrophosphatases
V-type	Vacuolar type
v/v	Volume-volume
VIT	Vacuolar iron transporter
W	Tryptophan
w/v	Weight-volume
WHO	World Health Organization
x	Times

X.	*Xenopus*
X-ray	Röntgen radiation
Y	Tyrosine
YPDA	Yeast extract-peptone-dextrose-adenine
ZFNL	Left zinc-finger nuclease
ZFNR	Right zinc-finger nuclease
ZFNs	Zinc-finger nucleases
α	Anti
µ	Micro
°C	Degree Celsius

1. Introduction

1.1. Malaria etiology, symptoms and epidemiology

Malaria is an infectious disease caused by parasitic protozoa from the genus *Plasmodium* and transmitted by female mosquitoes of the genus *Anopheles*. In humans, malaria is caused by the species *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale (P. ovale curtisi and P. ovale wallikeri)* and *P. knowlesi* (Sutherland et al., 2010). *P. falciparum* is the species that causes the highest number of deaths worldwide. However, outside sub-Saharan Africa, *P. vivax* causes half of the malaria cases and is responsible for between 3.5% and 16% of the total deaths. *P. knowlesi* can also cause severe malaria and death, although to a minor extent (WHO, 2015a). Additionally, *P. vivax* can cause multiple relapses of the disease since it can remain dormant in infected liver cells in a cellular stage known as hypnozoite (Imwong et al., 2007; Krotoski et al., 1986). *P. ovale* can as well cause relapses of the disease, but there is no biological evidence proving that this species also generates hypnozoites (Richter et al., 2010). However, hypnozoites have been found in monkeys infected with *P. simiovale*, an analog of the human parasite *P. ovale* (Cogswell et al., 1991).

The first symptoms of malaria are fatigue, headache and muscle pain followed by fever, shivering and vomiting. When the patient receives appropriate treatment at this stage, the prognosis is good. But when *P. falciparum* infections remain untreated, they might develop into severe malaria, which can still be cured but is lethal in the majority of cases if left untreated. The symptoms of severe malaria include unarousable coma, severe distress, seizures and severe anemia. Besides the treatment regime, the outcome of the disease also depends on the parasite strain and the immune status of the host (WHO, 2014).

More than 40% of the world's population (fig. 1.1) is at risk of being infected by *Plasmodium*. The various species have different geographical distributions; *P. falciparum* is predominant in sub-Saharan Africa and *P. vivax* in America and the western Pacific region. *P. knowlesi* is particularly prevalent in Malaysia, where it caused 38% of the malaria cases reported in 2014. In Southeast Asia the proportion of each species varies greatly between countries. Even in the eastern Mediterranean region, there are still six countries with high malaria transmission, mostly caused by *P. falciparum* (WHO, 2015a).

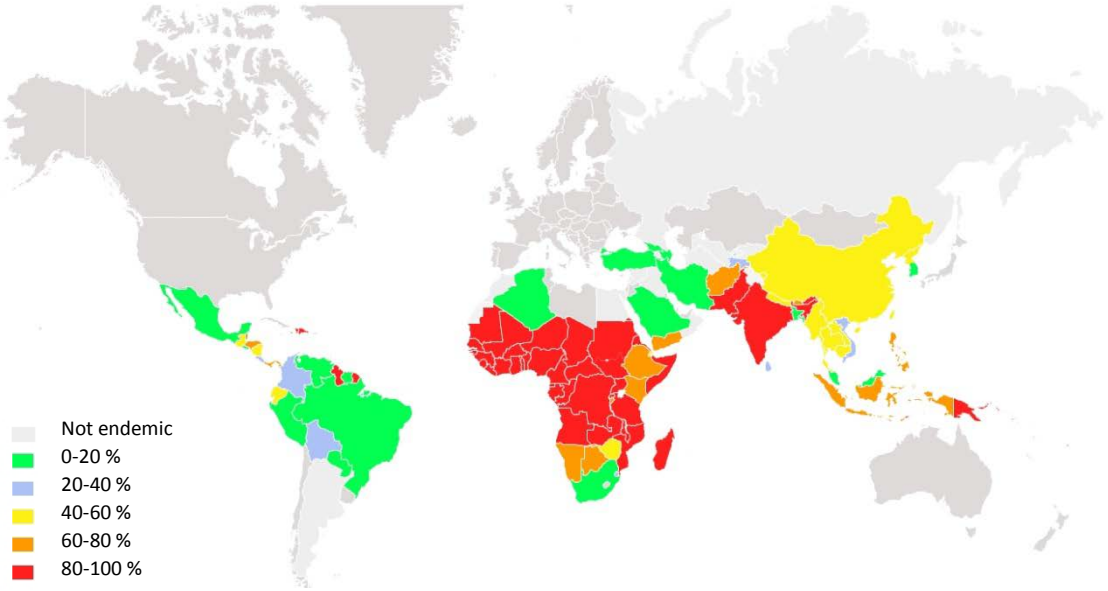


Figure 1.1. Percentage of population at risk of contracting malaria in 2013.
Map created with the online mapping tool "Global Malaria Mapper".

The fight against malaria has achieved remarkable progress during the last 15 years; malaria mortality has been reduced globally by 66% and particularly in children under five, by 71%. Malaria is no longer the highest cause of death among children under five in sub-Saharan Africa. This advance has been possible due to the increased use of insecticide-treated nets and a better access to diagnostic tests and appropriate treatments. Nevertheless, in 2015 there were still around 214 million new cases and 438,000 estimated deaths caused by malaria worldwide (WHO, 2015a).

1.2. *Plasmodium* biology

1.2.1. *Plasmodium* life cycle

Plasmodium has a complex life cycle; it multiplies asexually in the human host and sexually in the mosquito vector (fig. 1.2.A). Around 100 *Plasmodium* sporozoites are injected by a mosquito bite into the skin of the human host, where they become actively motile. Eventually, a few find a blood vessel, enter the bloodstream and get to the liver (Amino et al., 2006). Sporozoites traverse sinusoidal cells to reach the hepatocytes and they transmigrate through a few cells before they establish an infection (Frevert et al., 2005). The initial attachment step in liver entry is mediated by the binding of CSP to heparan sulfate

proteoglycans (Frevert et al., 1993). TRAP (Sultan et al., 1997), AMA1 (Silvie et al., 2004), TRSP (Labaied et al., 2007) and the two proteins P36 and P36p (Ishino et al., 2005) are also involved in the early steps of hepatocyte invasion, although the host receptors they interact with haven't been identified so far. On the other hand, the hepatocyte molecules CD81 (Silvie et al., 2003) and SR-B1 (Rodrigues et al., 2008) also play a crucial role in hepatocyte invasion. After infecting a hepatocyte, the sporozoite undergoes multiple rounds of division and produces tens of thousands of merozoites that within 6 to 10 days, depending on the *Plasmodium* species, are released from the liver cell into the lumen of the liver sinusoids within membranous structures called merosomes. Once in the lung vasculature, merosomes burst releasing the merozoites into the bloodstream (reviewed by Prudencio et al., 2006).

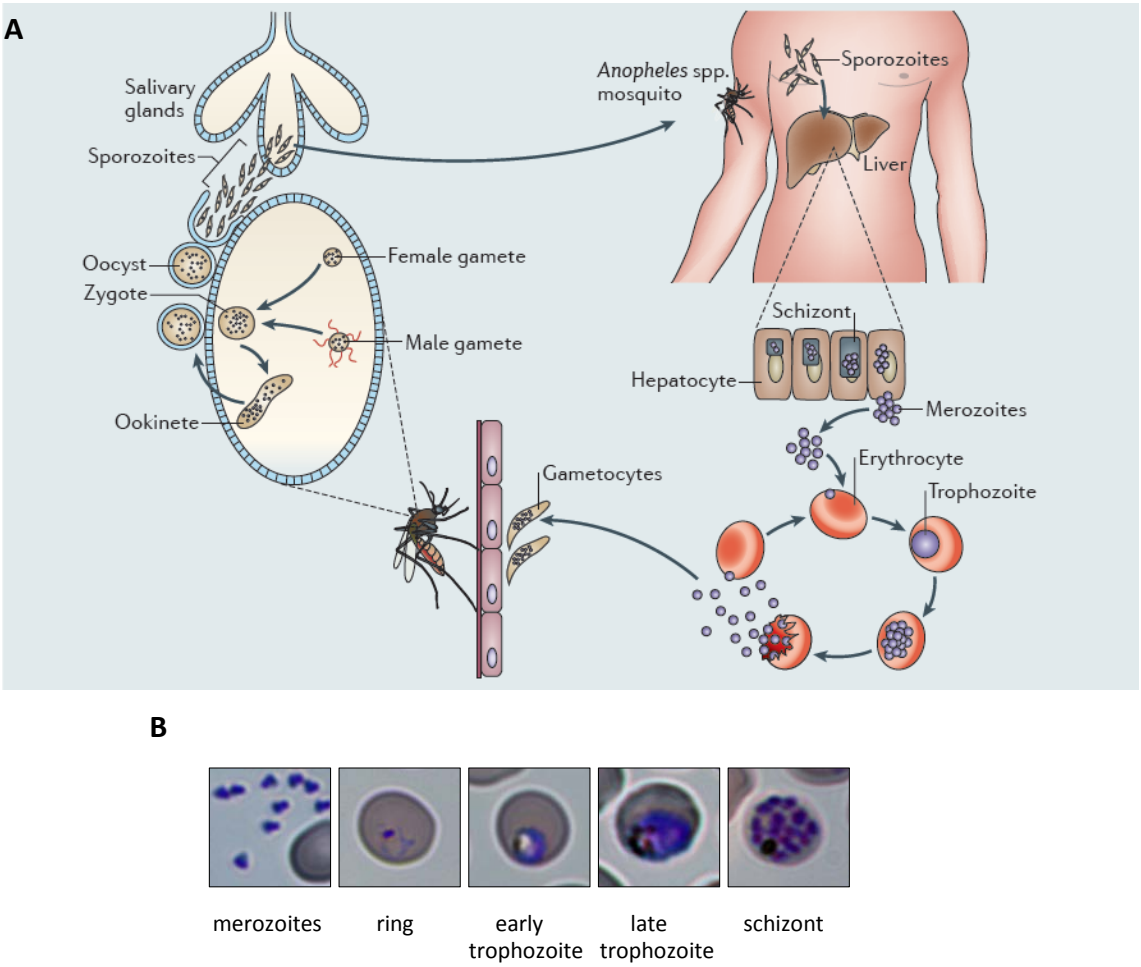


Figure 1.2. A. P. falciparum life cycle (de Koning-Ward et al., 2015). B. Images of Giemsa-stained P. falciparum blood stage parasites.

In the bloodstream, merozoites bind to and invade red blood cells (RBC). Erythrocyte binding is mediated by host receptors and parasite surface proteins mainly from the merozoite surface protein family; MSP1, for instance, is thought to bind the erythrocyte surface Band 3 (Goel et al., 2003). After egress and during invasion, the secretory organelles

called micronemes and rhoptries secrete parasite adhesins (EBL and PfRh families) and invasins (AMA1) at specific time points during the invasion process to ensure the correct sequence of attachment events (Singh et al., 2010). Merozoites can bind the RBC at any point on its surface but once they attach, they reorient with the apical end pointing to the RBC. A tight junction is then formed, creating an irreversible attachment event. At the tight junction, RON2, which is secreted by rhoptries and inserted into the erythrocyte membrane, links the parasite with the host cell membrane via its binding to AMA1 (Richard et al., 2010). Invasion is then driven by the actomyosin motor (Baum et al., 2006), with the tight junction moving along the merozoite surface, while the parasitophorous vacuole (PV) forms, until the merozoite pinches off (Riglar et al., 2011).

In *P. falciparum*, the intraerythrocytic cycle lasts 48h and causes the clinical symptoms associated with malaria (fig. 1.2.B). After invasion, the merozoite develops sequentially from ring to trophozoite to schizont. Each schizont then divides into 10-30 merozoites, the RBC ruptures and the merozoites are able to invade new erythrocytes. The first step in merozoite egress from the host cell is RBC destabilization, mediated by the proteases falcipain-2, plasmepsin II and the host calpain-1, which degrade several actin cytoskeleton proteins (Chandramohanadas et al., 2009; Hanspal et al., 2002; Le Bonniec et al., 1999). After cytoskeleton destabilization, PfSUB1 cleaves PfSERA5, which mediates the rupture of the parasitophorous vacuolar membrane (PVM) and therefore the release of free merozoites (Arastu-Kapur et al., 2008). Some of these proteases are stored at the exonemes, which discharge their content into the PV after egress is triggered by a yet unidentified signal (Yeoh et al., 2007).

During the intraerythrocytic cycle, the parasite remodels its host cell to a large extent by exporting proteins to the RBC. Exported proteins need to cross the plasma membrane of the parasite and the PVM in order to reach the RBC cytosol, and in some cases the RBC plasma membrane. Most of the exported proteins contain an export element (PEXEL) motif, the canonical signal that targets *P. falciparum* proteins for export to the host cell (Marti et al., 2004). This PEXEL sequence is acetylated and cleaved in the ER (Chang et al., 2008) before proteins are transported in vesicles to the PV. Protein translocation is mediated by the PTEX complex from the PV to the RBC cytoplasm (Beck et al., 2014; Elsworth et al., 2014). In order to establish its own protein export machinery in the RBC cytoplasm, the parasite creates the membrane structures called Maurer's clefts during or shortly after

invasion (Gruring et al., 2011). Exported proteins are thought to be transported in vesicles attached to actin filaments from this organelle to the knobs, electron-dense surface protrusions at the erythrocyte plasma membrane (Cyrklaff et al., 2011). It has also been postulated that Maurer's clefts are connected to the erythrocyte membrane via tubular structures where MAHRP2 is localized (Pachlatko et al., 2010).

The ligands expressed at the RBC membrane, mainly proteins from the RIFIN, STEVOR and PfEMP1 families, bind to the endothelium. This allows the parasite to cytoadhere and to evade the host immune response, causing the severe outcome of the disease (Niang et al., 2009; Smith et al., 1995). The PfEMP1 protein family is coded by the clonally variant *var* gene family, which enables the parasite to express different surface proteins over time as a second mechanism of immune evasion. In *P. falciparum*, only one *var* gene is expressed at a particular time point, whereas the other members of the family are silenced (Scherf et al., 1998). *Var* gene expression is regulated epigenetically through reversible histone modifications (Lopez-Rubio et al., 2007; Lopez-Rubio et al., 2009) and transcriptionally through non-coding RNAs (Swamy et al., 2011).

Some of the parasites (<10%) commit to sexual forms called gametocytes. Gametocyte commitment is a biological process which is poorly understood, although significant progress has been made in recent years. Schizonts are the stage that commit to sexual forms, producing merozoites that, upon reinvasion of new RBCs, will develop to gametocytes (Bruce et al., 1990). In *P. falciparum*, gametocyte development lasts 10-12 days and is divided into five stages. However, only stage I and stage V gametocytes are found in the bloodstream since the other stages sequester in the bone marrow (Joice et al., 2014). The disruption of the transcription factor AP2-G results in the downregulation of many genes expressed during the early stages of gametocyte development and therefore, causes the inability of the parasite to produce gametocytes (Kafsack et al., 2014; Sinha et al., 2014). The current model for regulation of sexual commitment is that, due to environmental signals or in a stochastic manner, those parasites that develop into gametocytes express AP2-G, whose expression activates early stage gametocyte genes triggering gametocytogenesis. AP2-G itself is thought to be regulated by epigenetic factors and to be silenced in the majority of the cells (Josling and Llinas, 2015).

Gametocytes are taken up by the mosquito with the blood meal, where they mature and develop into one female macrogamete or several male microgametes. In the mosquito

midgut, the gametes fuse to produce a zygote. The zygote develops to ookinete and invades the mosquito midgut wall, where it matures into an oocyst. Thousands of new sporozoites are formed within the oocyst that eventually ruptures, releasing the sporozoites, which migrate and invade the salivary glands of the mosquito. When the mosquito feeds from a new host, the cycle starts again.

1.2.2. Hemoglobin degradation and heme detoxification

During the intraerythrocytic cycle, the parasite consumes 60-80% of the RBC hemoglobin (Krugliak et al., 2002). Hemoglobin digestion starts at the late ring stage, when the erythrocyte cytoplasm is engulfed via cytostome-derived invaginations. The small vesicles generated merge and form a mature food vacuole when the trophozoite stage begins (Abu Bakar et al., 2010). Hemoglobin is digested in the food vacuole by different proteases, including falcilysin and members of the plasmepsin and falcipain families (Banerjee et al., 2002; Eggleson et al., 1999; Sijwali et al., 2006). The proposed digestion pathway is as follows: first plasmepsins truncate the native hemoglobin and then, falcipains cleave the denatured globin. After that, falcilysin recognizes these peptides of 10-15 amino acids long and generates small peptides that are further hydrolyzed to amino acids by the parasite's aminopeptidases (Gavigan et al., 2001; Gluzman et al., 1994). It has recently been suggested that plasmepsins and falcipains form a protein complex together with a heme detoxification protein that promotes hemozoin formation (Chugh et al., 2013).

During hemoglobin degradation, free heme is released and its iron molecule is oxidized from Fe^{+2} to Fe^{+3}. The resulting ferriprotoporphyrin IX (FP) is highly reactive and forms oxygen-free radicals, causing protein and DNA oxidation and lipid peroxidation when not detoxified (Orjih et al., 1981). The parasite forms hemozoin, a cyclic dimer of FP (Pagola et al., 2000) in order to avoid free FP's toxicity. There are different reports regarding the percentage of FP that is converted to hemozoin. Some studies showed a conversion of 30-50% (Ginsburg et al., 1998; Zhang et al., 1999) while others reported a conversion rate of above 80% (Egan et al., 2002). The proportion of FP that is not converted into hemozoin is hypothesized to be detoxified by glutathione (Ginsburg et al., 1998) and hydrogen peroxide (Brown et al., 1978).

It is not clear how hemozoin formation is initiated. It could be mediated by lipids, since hemozoin formation occurs within lipid nanospheres (Pisciotta et al., 2007), or by the

heme detoxification protein (DHP), a likely essential protein localized at the food vacuole that is highly efficient promoting hemozoin formation *in vitro* (Jani et al., 2008).

1.2.3. *P. falciparum* transporters

Transporters can be classified in two main categories: channels, which form aqueous pores and transport specific solutes across the membrane, and carriers, which bind to specific substrates and undergo conformational changes to mediate transport. Carriers can transport their substrates down their electrochemical gradient (facilitative carriers) or against it (pumps) using ATP (primary active carriers) or an ion gradient as a source of energy (secondary active carriers). More than 100 genes in the *P. falciparum* genome have been classified as putative transporters (Martin et al., 2005) of which only a few have been characterized up to date.

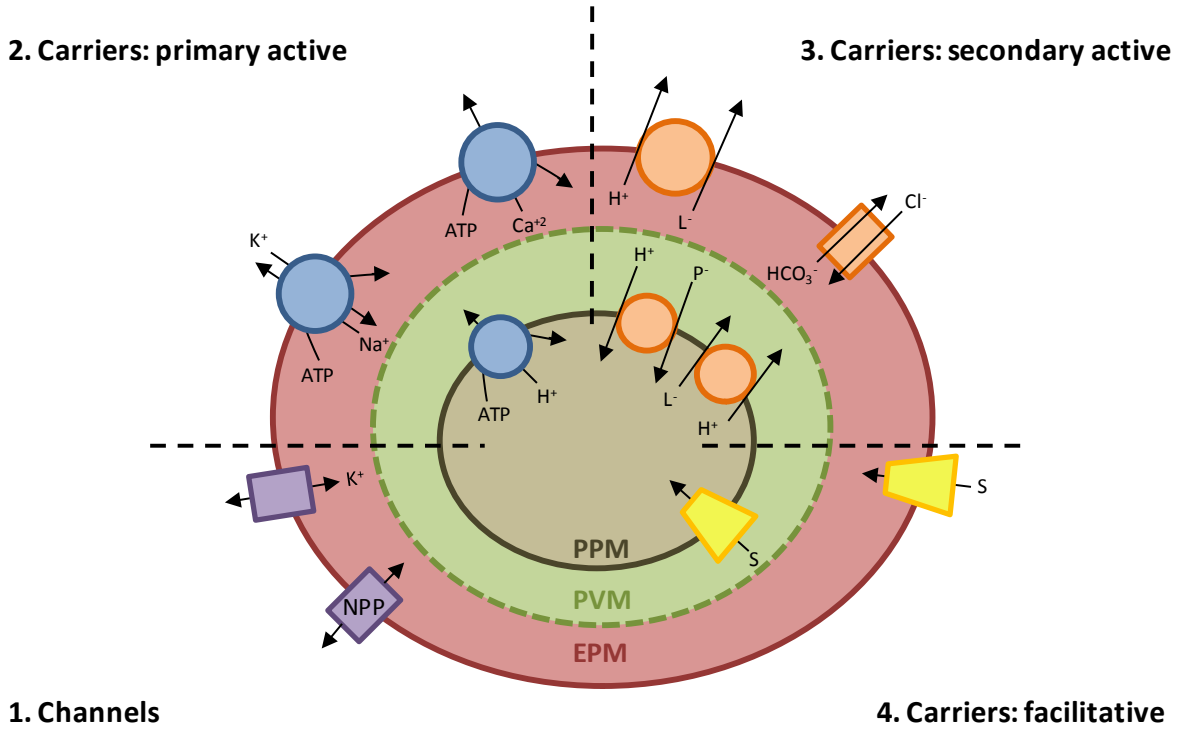


Figure 1.3. Schematic illustration depicting some representative transporters from P. falciparum and the iRBC (Staines et al., 2010).

The examples represented are classified according to the major classes of membrane transport proteins. Transporters are represented at the erythrocyte plasma membrane (EPM), parasitophorous vacuolar membrane (PVM) and parasite plasma membrane (PPM). Channels: new permeation pathways and the Ca^{2+}-activated K^+ channel. Primary active carriers: the Na^+/K^+ ATPase, the Ca^{2+} pump and a putative v-type H^+ pump. Secondary active carriers: the H^+ coupled monocarboxylate symporter that mediates the efflux of lactate (L^-), the HCO_3^-/Cl^- exchanger and the putatives H^+ coupled pantothenate (P^-) symporter and H^+ coupled lactate (L^-) symporter. Facilitative carriers: the hexose transporters GLUT1 and PfHT.

One of the first *P. falciparum* transporters to be studied was PfHT, an essential hexose carrier that belongs to the major facilitator superfamily (MSF) (Woodrow et al., 1999). Putative transporters of this family, for instance the putative organic cation transporter PFE0825w, are predicted to mediate the transport of folate, choline, lactate or pantothenate, but there is, to date, no experimental evidence to support this assumption. In *P. falciparum* there are 12 proteins that belong to this family (Martin et al., 2005), a small proportion compared with other living organisms which exhibit around 100 members. The parasite genome also codes for several anion and cation carriers, such as PfPiT, a sodium/phosphate cotransporter that localizes to the parasite plasma membrane (Saliba et al., 2006) or the recently identified vacuolar iron-transporter (PfVIT) that localizes to the parasite endoplasmic reticulum. Although VIT is not essential in *P. berghei*, PbVIT knockout parasites are more sensitive to high concentrations of iron, suggesting that one of the roles of this transporter is iron detoxification (Slavic et al., 2016).

Among the *P. falciparum* putative pumps, there are two belonging to the P-type ATPases which have been partially characterized. PfATP4 is a cation ATPase localized at the plasma membrane and at undefined membrane structures within the parasite (Rottmann et al., 2010) while the PfCuP ATPase is a putative copper transporter localized at the parasite and host plasma membranes (Rasoloson et al., 2004). There are also 12 subunits of a *v-type* H^+ *ATPase*, 2 *v-PPase* genes and the subunits of a mitochondrial *f-type* H^+ *ATPase* present in the *P. falciparum* genome that could be potential drug targets but haven't been investigated so far.

Regarding *P. falciparum* channel transporters, PfAQP is one of the 12 putative channels, and it plays an important role in the parasite biology. PfAQP has been linked to osmotic protection, glycerol uptake, oxidative stress reduction and detoxification (Hansen et al., 2002; Pavlovic-Djuranovic et al., 2006). Among the 12 putative channels, there are also 4 K^+ channels, 2 of which have been partially characterized. Pfkch1/PfK1 localizes at the RBC plasma membrane and Pfkch2/PfK2 at the parasite plasma membrane and both might be essential in blood stages (Waller et al., 2008).

Nucleoside and amino acid transporters also play an essential role during blood stage development. In order to survive, the parasite needs to take up several nutrients such as pantothenic acid (Saliba et al., 1998) or isoleucine, the only amino acid that is not present in hemoglobin (Liu et al., 2006). Only PfNT1, 1 of the 4 predicted *P. falciparum* nucleoside

transporters, has been characterized; it is essential and localizes to the parasite plasma membrane (El Bissati et al., 2008). On the other hand, none of the 6 putative amino acid transporters of the parasite have been studied so far.

P. falciparum transporters that localize to the mitochondria or the apicoplast organelles have also been identified. Two ATP/ADP translocases which show mitochondrial localization have been expressed and characterized in *E. coli* (Razakantoanina et al., 2008) and the apicoplast PfiTPT and PfoTPT transporters have been proposed to transport phosphorylated C3 compounds into the plastid (Mullin et al., 2006).

Although *P. falciparum* transporters are involved in multiple metabolic pathways and are essential in some cases for the uptake of nutrients and the discard of toxic metabolites, they haven't been exploited yet as antimalarial targets in part due to the absence of structural data. PfAQP is the only *P. falciparum* transporter with a resolved crystal structure (Newby et al., 2008). Furthermore, the cation ATPases PfATP6 and PfATP4 are the only transporters that have been validated as antimalarial targets (Jimenez-Diaz et al., 2014; Pulcini et al., 2013). PfATP6 has been proposed to be one of the molecular targets of artemisinins (Eckstein-Ludwig et al., 2003), although this hypothesis has been disputed (O'Neill et al., 2010b) and hasn't been confirmed by whole-genome sequencing of artemisinin resistant strains in more recent studies (Ariey et al., 2014).

Besides being relevant potential drug targets, transporters have also been investigated with regards to their role in drug resistance. Duplications or point mutations within *P. falciparum* transporters cause reduced drug susceptibility by reducing the drug concentration in the compartment where the drug exerts its antimalarial activity. The main transporters that cause drug resistance in the parasite are PfCRT (described later), PfMDR1, PfMRP and PfNHE.

PfMDR1 is an ATP-binding cassette (ABC) transporter with 12 predicted transmembrane domains that localizes to the food vacuole with both termini facing the cytosol (Cowman et al., 1991; Karcz et al., 1993). The wild type protein transports the antimalarial drugs quinine and chloroquine and the mutant form transports halofantrine (Sanchez et al., 2008a). Its amplification confers resistance to lumefantrine, artemisinin, quinine, mefloquine and halofantrine (Sidhu et al., 2006) and several point mutations ($N^{86}Y$, $Y^{184}F$, $S^{1034}C$, $N^{1042}D$ and $D^{1246}Y$) influence the parasite susceptibility to lumefantrine,

artemisinin, quinine, mefloquine, halofantrine and chloroquine (Reed et al., 2000; Sidhu et al., 2005).

Two other ABC transporters have been associated with drug resistance, PfMRP1 and PfMRP2. The first one localizes to the plasma membrane and membrane-bound vesicles (Klokouzas et al., 2004). It is not essential, but when knocked out, the parasite susceptibility to chloroquine, quinine, artemisinin, piperaquine and primaquine increases (Raj et al., 2009). Point mutations within this transporter ($Y^{191}H$ and $A^{437}S$) are also associated with increased resistance to chloroquine and quinine (Mu et al., 2003). Likewise, a deletion in the 5'UTR region of the *pfmrp2* gene also correlates with increased levels of resistance towards chloroquine, quinine and mefloquine (Mok et al., 2014).

Differences in the copy number of the DNNND repeat (Ferdig et al., 2004) and expression levels (Nkrumah et al., 2009) of the PfNHE Na^+/H^+ exchanger have been associated with quinine resistance, although this association remains controversial (Andriantsoanirina et al., 2010). The transporter localizes to the parasite plasma membrane and has 12 predicted transmembrane domains (Bennett et al., 2007).

1.3. Malaria treatment and resistance

1.3.1. Antimalarial drugs and their targets

The bark from the cinchona tree was introduced into Europe as antimalarial treatment in the 17th century by Jesuit priests returning from Peru. The active compounds of this plant are quinine and its diastereomer quinidine. Nowadays, quinine is only recommended in combination with the antibiotic clindamycin to treat women in the first trimester of pregnancy (WHO, 2015b). Quinidine is more active than quinine but its use has been associated with severe side effects.

Efforts to produce synthetic quinine led to the discovery of the 4-aminoquinolone chloroquine (CQ) in 1934, which was used worldwide until resistance emerged around 1960. CQ is able to diffuse across membranes when it is not protonated, but in the acidic digestive vacuole pH, CQ is protonated and becomes membrane impermeable thus, accumulating in this organelle (Yayon et al., 1984). It is generally accepted that CQ inhibits heme detoxification by binding to FP and therefore blocking hemozoin formation (Bray et al., 1998; Chou et al., 1980). However, it has also been suggested to block hemoglobin degradation by

reducing its binding affinity to falcipain-2 (Chugh et al., 2013). CQ is currently still used to treat *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* infections, although *P. vivax* CQ resistance has already been confirmed in ten countries around the world (WHO, 2015a).

Several CQ derivatives such as amodiaquine and piperaquine were developed in order to overcome CQ resistance and they are used nowadays in artemisinin combination therapies (ACTs). Based on structural similarity, amodiaquine and piperaquine are thought to share the same mode of action as CQ. Although amodiaquine is effective against CQ resistant strains it also shows some cross-resistance (Sa et al., 2009). Piperaquine efficacy is also reduced against strains carrying CQ resistant PfCRT haplotypes, although to a lesser extent (Muangnoicharoen et al., 2009).

Arylamino alcohols derived from the chemical structure of quinine such as mefloquine, halofantrine and lumefantrine are also used to treat malaria. Mefloquine is used in prophylaxis and in combination with artesunate, despite its psychiatric side effects, since it is active against CQ-resistant strains (Tansley et al., 2010). Mefloquine resistance is associated with an increased concentration of the drug in the food vacuole, suggesting that it inhibits a cytosolic drug target. On the other hand, mefloquine inhibits the PfMDR1-mediated transport of other compounds into the food vacuole and therefore, the transporter has also been proposed as its potential target (Rohrbach et al., 2006; Sidhu et al., 2006). Furthermore, an alternative hypothesis suggests that mefloquine's mode of action is endocytosis inhibition (Hoppe et al., 2004). Halofantrine is also active against CQ-resistant strains and probably shares the same mode of action and mechanism of resistance with mefloquine but it is currently not used due to its serious side effects. Conversely, lumefantrine is well tolerated and it is currently used in a combination therapy with artemether.

Another antimalarial drug currently in use is atovaquone, which targets the cytochrome bc1 complex and consequently inhibits the electron transport chain and collapses the mitochondrial membrane potential (Fry and Pudney, 1992). As a result, pyrimidine biosynthesis is inhibited and the parasite dies (Painter et al., 2007). Atovaquone is only used in combination with proguanil and only as prophylactic treatment due to its high cost and because the parasite develops resistance quickly through mutations in the *cytb* gene.

Primaquine, an 8-aminoquinoline, is the only drug available that is effective against *P. vivax* hypnozoites and young stage gametocytes. It is used to reduce transmission in combination with ACTs (WHO, 2015b). However, it is not recommended to patients with glucose 6-phosphate 1-dehydrogenase (G6PD) deficiency due to an increased risk of hemolytic anemia (Bolchoz et al., 2001). One single dose of tafenoquine, a primaquine analog currently under development, could be as effective as 14 days of treatment with primaquine although it doesn't overcome the side effects in patients with G6PD deficiency. Tafenoquine is expected to be approved in the upcoming years (Price and Nosten, 2014). Their mode of action is not known, but primaquine doesn't inhibit heme polymerization like other quinolines (Hawley et al., 1998). No evidence of resistance against primaquine has been reported so far, instead it has been suggested that it can revert CQ resistance by inhibiting PfCRT (Bray et al., 2005; Sanchez et al., 2004).

Inhibitors of the dihydropteroate synthase (DHPS) and the dihydrofolate reductase (DHFR), both key enzymes of the folate biosynthetic pathway, have also been used as antimalarial drugs. The DHPS inhibitor sulfadoxine is used in combination with the DHFR inhibitor pyrimethamine, although only as ACT in combination with artesunate or as intermittent preventive treatment during pregnancy (WHO, 2015b).

In the current situation, the World Health Organization recommends the use of artemisinin-based combination therapies (an artemisinin derivative plus a second antimalarial) to treat uncomplicated *P. falciparum* malaria and parenteral artesunate or artemether to treat severe malaria. The partner drug should have a different mode of action and a longer half live than the artemisinin derivative in order to avoid the development of resistance. Artemisinin was also identified from a plant extract, in this case *Artemisia annua*, also known as sweet wormwood. It is a sesquiterpene lactone that exhibits an endoperoxide bridge essential for the antimalarial activity of the compound. Several artemisinin derivatives were synthesized in order to improve artemisinin solubility. These include dihydroartemisinin, artesunate and artemether, which are the fastest acting antimalarials known so far. Dihydroartemisinin (DHA), which is also an active metabolite of artesunate and artemether, inhibits the *P. falciparum* phosphatidylinositol 3-kinase (PfPI3K) (Vaid et al., 2010). There are other hypothesis regarding the mode of action of artemisinin; one claims that artemisinin derivatives inhibit PfATP6 (Krishna et al., 2014) while another claims that their mode of action is related to hemoglobin digestion (Klonis et al., 2013). The current

recommended ACTs include artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, dihydroartemisinin + piperaquine and artesunate + sulfadoxine-pyrimethamine.

1.3.2. Drug resistance mechanisms

Chloroquine resistance arose from 6 different loci in the Mekong region, Colombia and India in the late 50s and early 60s and it later spread to Africa (fig. 1.4) (Mehlotra et al., 2008; Wootton et al., 2002). Resistance to sulfadoxine-pyrimethamine arose in the 60s and to mefloquine in the 70s. In 2009, the first reports of artemisinin resistance appeared and recently also to piperaquine, marking the first time that an artemisinin combination therapy fails because the parasite develops resistance to both drugs (Amaratunga et al., 2016). Combination therapies reduce the chances of resistance emergence but when one of the drugs is not effective anymore, the likelihood of the parasite developing resistance towards the second drug increases, as in the current situation. Whether artemisinin resistance will spread to other geographical areas in the way that CQ resistance did four decades ago, is still unknown. So far, resistance to artemisinins has been confirmed in Cambodia, Laos, Myanmar, Thailand and Vietnam (WHO, 2015a).

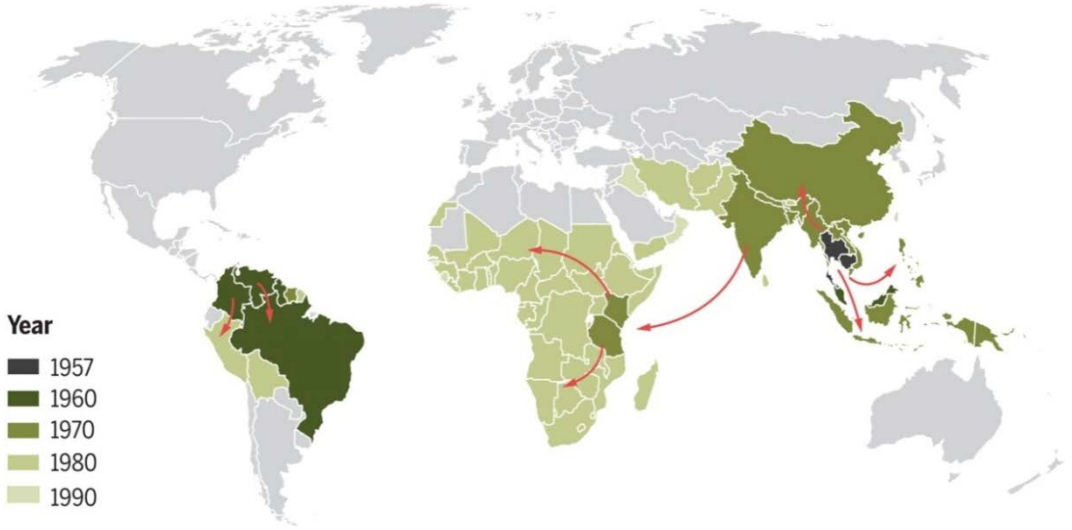


Figure 1.4. Spread of chloroquine resistance (Roberts, 2016).

CQ resistance is associated with mutations on the chloroquine resistance transporter (PfCRT) which promotes CQ transport out of the food vacuole, thus reducing the amount of

CQ available to bind its target (Sidhu et al., 2002). All CQ-resistant strains carry a mutation on the PfCRT residue K^{76}. The positive charge of the lysine side chain is thought to block the binding of CQ to the active site of the transporter in CQ-sensitive strains. Further mutations in PfCRT are thought to balance the level of parasite resistance and fitness. Interestingly, the mutation of serine 163 to arginine confers halofantrine resistance but restores CQ susceptibility (Johnson et al., 2004). Mutations in other transporters have also been linked to CQ resistance, as the mutation $N^{86}Y$ in PfMDR1 (Duraisingh et al., 1997) or the mutations $Y^{191}H$ and $A^{437}S$ in PfMRP1 (Mu et al., 2003).

Resistance against quinine, mefloquine, halofantrine, lumefantrine and artemisinin has been linked to an amplification of the PfMDR1 locus (Price et al., 2004). Mefloquine, halofantrine and artemisinin are transported by PfMDR1 into the food vacuole, thus preventing their activity against their putative targets in the cytosol (Rohrbach et al., 2006). In addition, the $N^{86}Y$ mutation of PfMDR1 confers resistance to lumefantrine (Sisowath et al., 2005). Quinine resistance has also been associated with point mutations in PfMDR1 (Sidhu et al., 2005), PfCRT (Cooper et al., 2007), PfNHE1 (Nkrumah et al., 2009) and PfMRP1 (Mu et al., 2003).

Resistance against DHFR inhibitors are directly linked to mutations in this protein. The sequential acquisition of mutations increases the level of resistance towards pyrimethamine and proguanil from 10-fold for the single mutant $S^{108}N$ to 500-fold for the quadruple mutant $S^{108}N/N^{51}I/C^{59}R/I^{164}L$. Similarly, resistance against sulfadoxine is due to mutations in the *dhps* gene. The mutations $S^{436}A/F$, $A^{437}G$, $K^{540}E$, $A^{581}G$ and $A^{613}T/S$ decrease the binding affinity of the inhibitor towards the enzyme (reviewed by Gregson and Plowe, 2005).

Mutations in the Kelch 13 propeller gene (*PfKelch13*) have been associated with reduced rates of parasite clearance and it is the only molecular marker for artemisinin resistance characterized so far (Ariey et al., 2014). Pfkelch3 mutations are spread in the five Southeast Asian countries where artemisinin resistance has been detected (fig. 1.5). It has been shown that dihydroartemisinin (DHA), the active metabolite of artemisinin, inhibits the phosphatidylinositol 3-kinase of *P. falciparum* (PfPI3K), which phosphorylates phosphatidylinositol (PI) and produces phosphatidylinositol 3-phosphate (PI3P) (Vaid et al., 2010). It has been proposed that PfKelch13 polyubiquitinates PfPIK3 in normal conditions

and that it fails to target PfPIK3 to the proteasome when mutated, leading to its accumulation and resulting in artemisinin resistance (Mbengue et al., 2015).

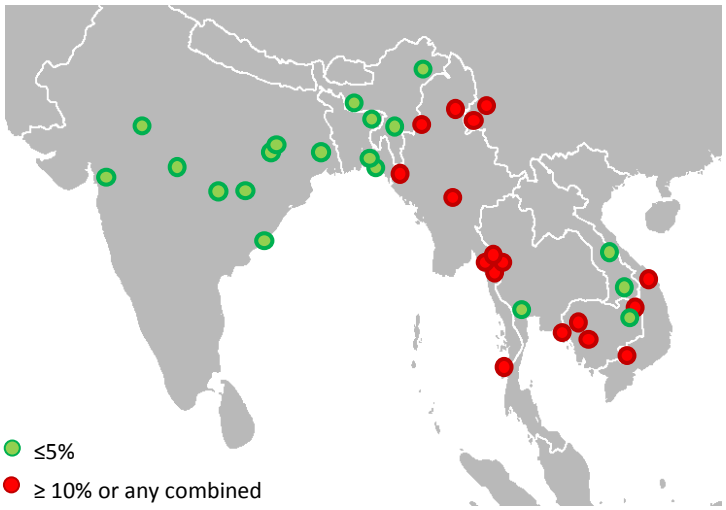


Figure 1.5. Current map distribution of PfKelch13 mutations. Adapted from the Worldwide Antimalarial Resistance Network (WWARN) website.

1.3.3. *P. falciparum* chloroquine resistance transporter

The *P. falciparum* chloroquine resistance transporter (PfCRT) belongs to the drug metabolite transporter (DMT) superfamily (Martin and Kirk, 2004). There is no crystal structure available, but it is predicted to have 10 transmembrane domains. It is localized at the digestive vacuolar membrane with both termini facing the cytoplasm (Fidock et al., 2000). Transport kinetics and trans-stimulation assays support the theory that PfCRT is a carrier and not a channel (Bellanca et al., 2014; Martin et al., 2009; Sanchez et al., 2007). The PfCRT locus was identified in a genetic linkage analysis of the F1 progeny of a genetic cross between the CQ-sensitive strain HB3 (CQS) and the CQ-resistant strain Dd2 (CQR) (Su et al., 1997). There are PfCRT homologs in *Plasmodium* and CRT-like proteins are also present in *Cryptosporidium parvum, Dictyostelium discoideum* and *Arabidopsis thaliana*, where the CRT-like protein transports γ-glutamylcysteine, a glutathione precursor (Maughan et al., 2010).

PfCRT is predicted to have an essential physiological function. Other members of the DMT family transport amino acids, weak bases and organic cations. In the case of PfCRT, many physiological substrates have been proposed but none has been confirmed. So far it

has been linked to the transport of amino acids or small peptides (Martin et al., 2009, Juge et al., 2015) and glutathione (Patzewitz et al., 2013).

In general, parasite strains with mutated PfCRT haplotypes accumulate less CQ than wild type strains (Fidock et al., 2000), mostly because they transport CQ out of the food vacuole (Martin and Kirk, 2004; Sanchez et al., 2005). This observation was confirmed in different parasite strains, genetically modified to express different *pfcrt* alleles in the same genetic background (Lakshmanan et al., 2005; Sidhu et al., 2002). PfCRT has also been heterologously expressed in different systems. $PfCRT^{Dd2}$ expressed in *Dictyostelium discoideum* resulted in decreased CQ and QN accumulation (Naude et al., 2005). Along the same line, oocyte studies showed that wild type PfCRT does not transport CQ but mutants do (Martin et al., 2009). Mutant haplotypes also alter the parasite susceptibility towards amodiaquine and lumefantrine (Cooper et al., 2007; Echeverry et al., 2007; Sisowath et al., 2009).

Two different pathways have been proposed to explain the evolution from CQS haplotypes to CQR, including mutations that have little effect on CQ transport. At least 2 mutations are necessary to confer low levels of CQ transport: K^{76} to a non-positively charged residue and N^{75} or N^{326} to a negatively charged amino acid. Other mutations in PfCRT may balance the level of parasite resistance and fitness since only four of the eight mutations of the Dd2 allele are required to mediate high levels of CQ transport (Summers et al., 2014). The idea that CQ-resistant strains may have a fitness cost is supported by the fact that once CQ is not used as treatment for a long time, the wild type strains seem to overgrow the resistant ones (Kublin et al., 2003; Wang et al., 2005).

1.3.4. The future of antimalarial drugs

There is, at present, no commercially available vaccine against malaria and the most developed vaccine candidate RTS,S/AS01 showed only modest efficacy in infants and children in the lasts clinical trials that took place in malaria endemic regions (Rts, 2015). This, together with the spreading of artemisinin resistance, highlights the need to develop new antimalarial drugs with new modes of action. It is of special concern that, besides the new artemisinin-based combination treatments approved in 2009, no new antimalarial drug has been launched into the market in the past decade (Wells et al., 2015).

Currently, there are several compounds with new modes of action that are undergoing clinical trials or preclinical development. For instance, PfATP4 is inhibited by the compounds AJ557733 and PA21A092 and has been recently validated as a new antimalarial target (Jimenez-Diaz et al., 2014; Vaidya et al., 2014). Other compounds in the early stages of the malaria pipeline are: MMV390048, an inhibitor of the PI(4) kinase (McNamara et al., 2013); P218, an inhibitor of the *Plasmodium* dihydrofolate reductase (Yuthavong et al., 2012); DSM265, an inhibitor of the dihydroorotate dehydrogenase (Coteron et al., 2011) and KAF156, an inhibitor of the cyclic amine resistance locus (Meister et al., 2011).

New synthetic endoperoxides such as OZ439 and OZ277 are also under development. Although they contain the same active group present in artemisinin, OZ439 has shown to be active against artemisinin-resistant strains and both compounds show a reduced reproductive toxicology compared with artemisinin in preclinical safety studies. The potential antimalarial activity of artemisone and RK 182 is also being investigated (Nagelschmitz et al., 2008; O'Neill et al., 2010a).

Additionally, a new generation of aminoquinolines is in the pipeline. Ferroquine is one of these compounds and so far has been shown to be active against chloroquine, amodiaquine and mefloquine resistant strains (Dubar et al., 2011). AQ13 is another aminoquinoline undergoing preclinical development (Mzayek et al., 2007).

On the other hand, there are several known drugs that have been used to treat other diseases that are now under clinical studies for malaria treatment. Among these compounds are methylene blue and trimethoprim. Also, the known antibiotic fosmidomycin, an inhibitor of the DXP reductoisomerase (Jomaa et al., 1999), is ongoing clinical trials in combination with piperaquine.

In a different approach, large compound libraries have been screened against *P. falciparum*. Among around 6 million compounds tested, more than 25,000 kill the parasite with an IC_{50} of around 1 µM (Avery et al., 2014; Gamo et al., 2010). KAE609, a PfATP4 inhibitor, is the most advanced compound that has been identified in one of these high throughput screens (Rottmann et al., 2010).

1.3.5. Lipid metabolism as antimalarial target

Choline enters the host cell by the residual erythrocytic choline carrier and the new permeation pathways (Kirk et al., 1991). Then, an unknown organic cation transporter

mediates choline transport inside the parasite (Biagini et al., 2004). Once inside the parasite, choline is converted to phosphatidylcholine via *de novo* cytidine diphosphate choline pathway that involves three enzymes: choline kinase, phosphocholine cytidylyltransferase and choline/ethanolamine phosphotransferase. It can also be synthesized from serine and ethanolamine precursors in an alternative pathway (Pessi et al., 2004). Phospholipid metabolism is considered a potential antimalarial target, although there are currently no approved antimalarial drugs targeting this pathway (Ben Mamoun et al., 2010).

Already in the late 90s, several choline analogs were developed in order to inhibit phospholipid metabolism in *P. falciparum* since phosphatidylcholine is the most abundant phospholipid in *P. falciparum* membranes (Ancelin et al., 1998). The lead compound T3, a bis-thiazolium salt also known as albitiazolium or SAR97276, is known to accumulate in the parasite food vacuole where it binds to heme (Biagini et al., 2003). However, the main mode of action is thought to be the inhibition of choline transport inside the parasite. T3 also inhibits the enzymes involved in phosphatidylcholine biosynthesis, although at higher concentrations (Wein et al., 2012). T3 entered clinical trials but was discontinued because it did not meet its primary endpoint in Phase II (Sanofi, 2013). Currently, efforts are focused on the design of albitiazolium prodrugs in order to improve the oral availability of the bis-thiazolium salts (Peyrottes et al., 2014).

1.4. Post-translational modifications in *Plasmodium*

1.4.1. Phosphorylation in *Plasmodium*

Post-translational modifications (PTMs) regulate all stages of *Plasmodium* species. Invasion, motility, exflaggellation, proliferation and egress are regulated by phosphorylation and lipidation (palmitoylation, myristoylation and prenylation). Furthermore, epigenetic regulation, which implies PTMs of histones, is involved in hypnozoite regulation, *var* gene expression and gametocytogenesis commitment (reviewed by Doerig et al., 2015).

The *P. falciparum* genome codes for around 90 kinases, of which 36 out of the 65 that have been studied are likely essential in blood stages (Solyakov et al., 2011). The majority of kinases are predicted to belong to the eukaryotic protein family; however, there are some discrepancies between the parasite and the human kinome. Tyrosine kinases are absent in *Plasmodium* and on the contrary, the FIKKs and CDPKs families are present in *Apicomplexa*

parasites but not in mammals. On the other hand, 30 protein phosphatases were identified in the *P. falciparum* genome (Wilkes and Doerig, 2008).

Among the characterized kinases, PfPKG is essential for merozoite egress. Its activity is necessary for exonemes and micronemes discharge (Collins et al., 2013b). Another essential kinase is PfPKA, which regulates invasion by phosphorylating AMA1 (Leykauf et al., 2010). *Plasmodium* kinases also play essential roles in non intra-erythrocytic stages. For example, PbCDPK4 and PbMAP2 control the process of male gametocyte differentiation into gametes (Billker et al., 2004; Tewari et al., 2005) and the *P. falciparum* and *P. berghei* eIF2α kinases regulate translation and play an essential role in stress-response and the transition of the parasite between different stages (Fennell et al., 2009; Zhang et al., 2010; Zhang et al., 2012).

1.4.2. Other post-translational modifications in *Plasmodium*

Much less is known about the role of palmitoylation, the reversible addition of a long-chain fatty acid to a cysteine residue, in *P. falciparum* biology. It is the second more abundant modification of parasite proteins in blood stages after phosphorylation. Proteins implicated in trafficking, cytoadherence, signaling, metabolism, invasion and drug resistance are palmitoylated, suggesting a crucial role of this modification in several essential pathways of the parasite. Palmitoylation inhibition by 2-bromopalmitate causes abnormal development of blood stage parasites and invasion impairment due to reduced levels of the complex motor proteins PfGAP45 and PfMTIP (Jones et al., 2012). Furthermore, disruption of the palmitoyltransferase PfDHHC9 leads to a decrease in gametocyte numbers. Attempts to disrupt PfDHHC3, 7 and 8 by double crossover recombination failed, suggesting an essential role of these palmitoyltransferases in blood stages (Tay et al., 2016).

Other lipidation modifications are myristoylation and prenylation, both irreversible. Myristoylated proteins have also been identified in *P. falciparum* blood stages and molecules targeting the only *P. falciparum N*-myristoyltransferase have proven to arrest the parasites at the schizont stage (Wright et al., 2014). Similarly, prenylation inhibitors cause mislocalization of Rab5, a small GTPase, which mediates cellular vesicular trafficking (Howe et al., 2013).

The main role of methylation and acetylation is to control transcription levels through histone modification. This regulatory mechanism is particularly important in *var* gene regulation (Freitas-Junior et al., 2005; Lopez-Rubio et al., 2009).

N- and *O*-linked glycosylation (Kimura et al., 1996; Nasir ud et al., 1992), ubiquitylation (Ponts et al., 2011), sumoylation (Issar et al., 2008), S-nitrosylation (Wang et al., 2014) and S-glutathionylation (Kehr et al., 2011) have also been detected in plasmodial proteins but their roles in *Plasmodium* biology have not been deeply investigated.

1.4.3. Transporters regulation by phosphorylation

No *Plasmodium* transporter has been reported so far to be regulated by phosphorylation. However, the cellular functions of multiple transporters have been shown to be regulated by phosphorylation in other eukaryotic organisms.

Phosphorylation can either activate or inhibit the activity of a transporter. For example, the activity of the rat organic cation transporter (rOCT1) is stimulated by the phosphorylation of a serine residue by protein kinase C (Mehrens et al., 2000). Conversely, phosphorylation of the dopamine transporter reduces its maximal velocity (Moritz et al., 2015). Phosphorylation can also regulate the cellular localization of a transporter, therefore regulating the levels of functional protein present at the membrane. This is the case of the water channel aquaporin-2 (Moeller et al., 2011) and the insulin-regulatable glucose transporter (Lawrence et al., 1990). Both transporters show increased levels of protein expression at the membrane when phosphorylated. On the contrary, when the glutamate transporter GLAST is phosphorylated by the glycogen synthase kinase 3 isoform β (GSK3β), the protein levels of this transporter at the membrane decrease (Jimenez et al., 2014).

Interestingly, transporters linked to drug resistance are also regulated by phosphorylation. This is the case with hMDR1, the human homolog transporter of the *P. falciparum* multidrug resistance transporter PfMDR1 (Aftab et al., 1994). In *Plasmodium*, PfMDR1 is phosphorylated at residues S14, S514 and T513 (Solyakov et al., 2011) but the biological functions of these post-translational modifications are not known.

The new permeation pathways (NPPs) induced by the parasite upon RBC infection (Krugliak and Ginsburg, 2006) are also thought to be regulated by phosphorylation. When the phosphoproteomes of RBC and iRBC are compared, 5 membrane proteins (PMCA, AQ1, Band3, GLUT1 and the nucleoside transporter 1) appear to be differentially phosphorylated

(Bouyer et al., 2016). Infected RBCs are more permeable to inorganic anions and cations, sugars, amino acids, peptides and nucleosides, among other compounds. The increased permeability of the iRBC is due to the expression of parasite anion transporters from the *clag* gene family at the host cell membrane (Nguitragool et al., 2011) as well as due to an upregulation of the RBC endogenous channels (Staines et al., 2007). The exact mechanism of activation is still under discussion. On the one hand, RBCs subjected to oxidative stress mimic the induced hemolysis and electrophysiological properties of iRBCs (Huber et al., 2002) but on the other hand, protein kinase A can also activate RBC anion channels (Egee et al., 2002).

1.4.4. Post-translational modifications of PfCRT

PfCRT phosphorylation at residues 33 and 411 has been experimentally proven in different studies but the functional role of these modifications still remains to be elucidated (Kuhn et al., 2010; Lasonder et al., 2012; Solyakov et al., 2011). Phosphorylation of serine 420 was also detected in one of these studies (Lasonder et al., 2012). There is evidence that PfCRT localization is regulated by this post-translational modification. The threonine residue at position 416 is essential for the trafficking and localization of the transporter to the food vacuole. When the residue T^{416} of an episomal copy of PfCRT tagged with GFP is mutated to alanine, the transporter is localized at the plasma membrane. This phenotype can be partially rescued by substitutions with aspartic and glutamic acid (Kuhn et al., 2010).

Another post-translational modification that takes place in PfCRT is the palmitoylation of the cysteine residue 301. This modification was detected in a large-scale study that identified more than 400 blood stage palmitoylated proteins (Jones et al., 2012). The role of palmitoylation in PfCRT function remains to be investigated. However it is possible to speculate that palmitoylation at position 301 may change the topology of PfCRT by promoting the insertion of the outside loop into the membrane.

1.5. Molecular tools in *P. falciparum*

1.5.1. Genome editing in *P. falciparum*

A huge progress has recently been made in the field of genome editing in *Plasmodium* with the implementation of the CRISPR-Cas9 technology (Ghorbal et al., 2014; Zhang et al., 2014). Before the introduction of this technology, to disrupt a gene or to do

allelic exchange required the transgenic parasite cultures carrying an episomal plasmid to be subjected to multiple rounds of ON/OFF drug selection, hoping for the plasmid to integrate by single (Crabb and Cowman, 1996; Wu et al., 1996) or double crossover recombination (Duraisingh et al., 2002; Maier et al., 2006).

The CRISPR-Cas9 technology is based on a prokaryotic defense system that uses short RNAs to target and degrade foreign nucleic acids (Jinek et al., 2012; Mali et al., 2013). In the adapted system, the Cas9 endonuclease binds to a single guide RNA (sgRNA) that is designed to be complementary to a specific sequence of the gene of interest. Cas9 introduces a double strand break into the genome that is repaired by homologous recombination using a DNA template that has been modified to introduce the desirable genetic modification (fig. 1.6.A).

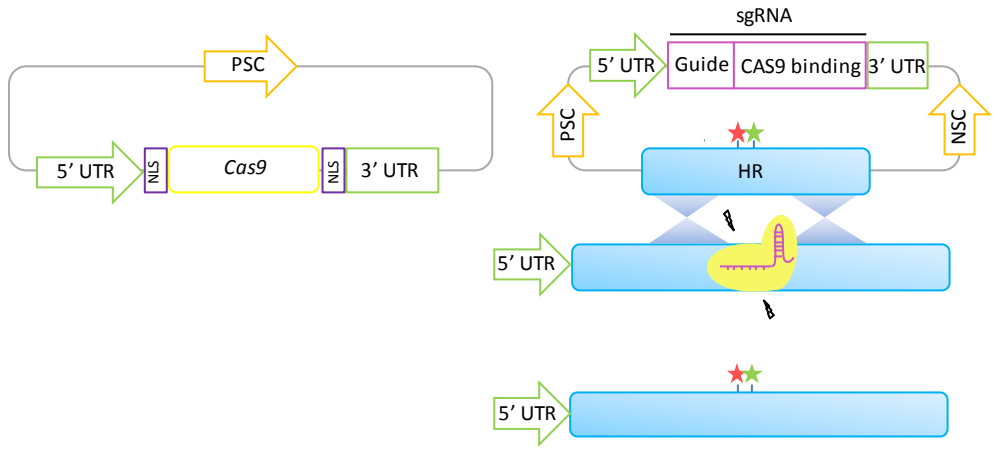
The use of zinc-finger nucleases (ZFNs) is an alternative methodology that also introduces double strand breaks into the genome using site-specific nucleases (fig. 1.6.B). This technology has been adapted to the malaria parasite (Moraes Barros et al., 2015; Straimer et al., 2012) but due to the high costs derived from the complex DNA-binding domain design process, it is not widely used.

Both methodologies depend on DNA double-strand break repair (DSBR). There are two main mechanisms of DSBR in eukaryotes: homologous recombination (HR) and end joining (EJ). EJ can be further divided into the classical non-homologous end joining (NHEJ), and the two alternative pathways, microhomology-mediated end joining (MMJE) and single strand annealing (SSA). HR is a high fidelity mechanism, since homology sequences of DNA are used to repair the breaks. On the contrary, EJ pathways usually cause deletions or insertions because the broken DNA is just ligated together (NHEJ) or by annealing of homology sequences that are exposed after the DSB (MMJE and SSA). In *P. falciparum*, the genes involved in NHEJ have not been identified, suggesting that the parasite lacks this repair mechanism (Lee et al., 2014). On the other hand, *P. falciparum* has the molecular machinery necessary to complete alternative EJ pathways although these repair events are not frequently detected (Straimer et al., 2012). This fact makes the genome editing techniques based on DSB repair by HR especially suitable for *P. falciparum*.

Both ZFNs and CRISPR-Cas9 make it possible to mutate, disrupt, replace and tag genes without altering the genomic region by introducing a selectable marker or creating

partial duplication of the gene of interest as a result of a single or double recombination. Nevertheless these modifications are permanent and cannot be modulated.

A. CRISPR-Cas9



B. Engineered Zing Finger Nucleases

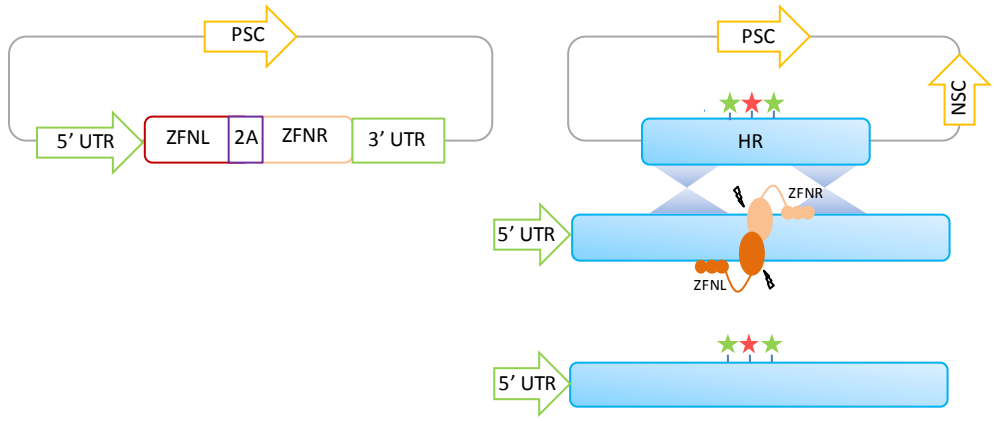


Figure 1.6. Site specific genome editing strategies currently available in P. falciparum.

1.5.2. Gene downregulation strategies in *P. falciparum*

There are several established methodologies to knockdown or conditionally knockout genes in *P. falciparum*: the tetracycline-repressible transcriptional system, the PKBP12 destabilization domain (DD), the *E. coli* DHFR degradation domain (DDD), the conditional aggregation domain (CAD), the glmS ribozyme and the Cre-*loxP*, FLP-*frt* and diCre systems (reviewed by de Koning-Ward et al., 2015). The use of interference RNA (RNAi) is not possible in *P. falciparum* because the necessary enzymes are not present in the parasite's

genome (Baum et al., 2009). Another methodology that could be used, but hasn't been reported in *P. falciparum* so far, is the use of a mutant version of Cas9 to block transcription (Gilbert et al., 2013).

The first approach which showed that it is possible to regulate gene expression in *P. falciparum* was the Tet-OFF system. This strategy uses anhydrotetracycline (ATc) - regulated transactivators, identified in *T. gondii*, to control the expression of *P. falciparum* genes. The promoter of the target gene has to be modified to incorporate tetracycline operator sites (TetO). In absence of ATc, the transactivators bind to the TetO sites and promote the transcription of the gene. When ATc is added, the gene expression is repressed since the transactivators are sequestered by ATc and cannot bind to the TetO sites. Unfortunately, this system only enables the regulation of episomally expressed transgenes (Meissner et al., 2005).

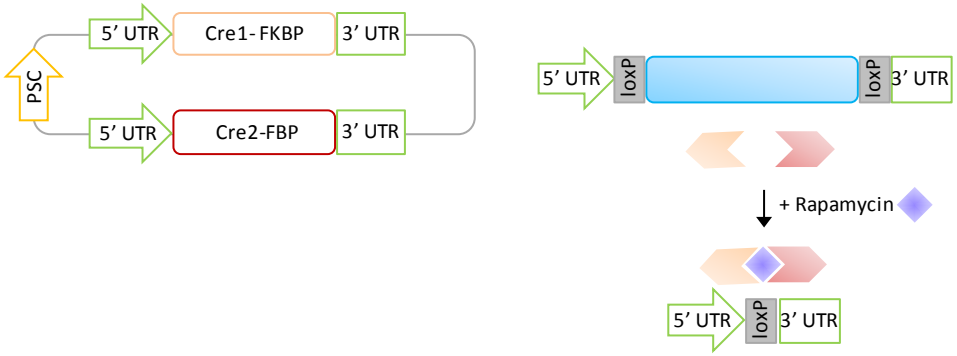
The Cre-*loxP*, FLP-*frt* and diCre systems are based on the directed recombination of two short sequences (*loxP* or *frt*) flanking the gene of interest by an inducible recombinase (Cre or FLP) (O'Neill et al., 2011). In the case of diCre, the recombinase is expressed in two different subunits that aggregate and form a functional heterodimer upon the addition of rapamycin (fig. 1.7.A). The last approach is the most efficient and allows the tightest regulation of recombinase activity. The main disadvantage is that recombination doesn't occur in the whole population of parasites, so it is problematic to link a particular phenotype directly to a knockout gene (Collins et al., 2013a).

The DD tag, when fused to a protein, targets the protein for proteosomal degradation. Protein levels are regulated via the addition of the compound Shield-1, which stabilizes the protein and prevents its degradation (fig. 1.7.B). Its main disadvantage is that the DD tag can interfere with the function of the tagged protein, making the tagging unfeasible (Armstrong and Goldberg, 2007). The PKBP12 destabilization domain (DD) is preferred over the *E. coli* DHFR degradation domain (DDD) since the compound trimethoprim used to stabilize the DDD domain is toxic for the parasites, which need to express hDHFR to survive (Muralidharan et al., 2011).

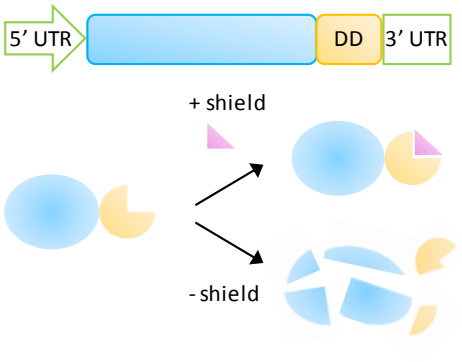
One strategy used to downregulate gene expression at the post-transcriptional level is the glmS ribozyme. In the adapted system, the glmS ribozyme sequence is cloned at the 3'UTR of the gene of interest. Upon addition of glucosamine, the glmS ribozyme cleaves its own mRNA. Consequently, both mRNAs are degraded, leading to a decrease in protein levels

of the targeted gene (fig. 1.7.C) (Prommana et al., 2013). The disadvantages of this system are the toxicity of glucosamine at high concentrations and poor levels of downregulation compared with other systems.

A. DiCre conditional gene deletion system



B. Destabilization domain (DD)



C. glmS ribozyme

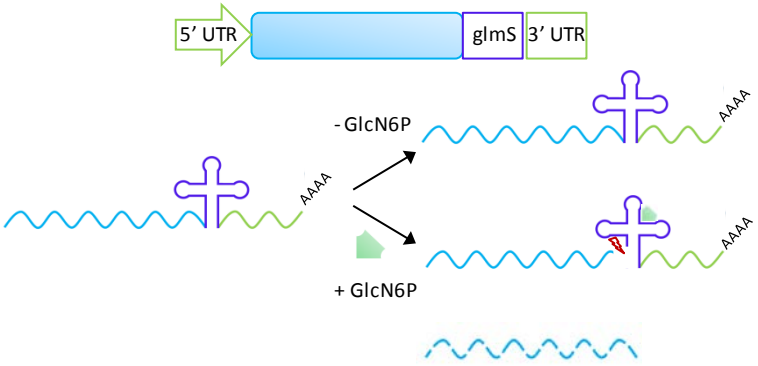


Figure 1.7.Knockdown strategies currently available in P. falciparum.

Another inducible system is the conditional aggregation domain (CAD) which aggregates in absence of the anti-aggregation ligand Shield-1. However, only secreted proteins have been shown to be regulated by this domain (Rivera et al., 2000; Saridaki et al., 2008).

A different strategy, the Tet repressor (TetR)-aptamer system, to post-transcriptionally downregulate protein levels was recently published. The TetR-aptamer is designed to bind specifically to the mRNA of the gene of interest, blocking translation. The addition of tetracycline disrupts this interaction, and promotes protein expression. No genome modification is necessary, which is one of the main advantages of the technique. Whether this system would be widely applicable in *P. falciparum* remains to be demonstrated (Goldfless et al., 2014).

1.6. *X. laevis* oocytes as a system to study membrane proteins

Xenopus oocytes are a well-established system that has been extensively used to study all major classes of transporters from different eukaryotic organisms. It was first used in the early 70s to synthesize rabbit hemoglobin (Gurdon et al., 1971) and some years later was used to express plant proteins, particularly maize proteins (Larkins et al., 1979). It has been proven to be a useful system to express proteins that require post-translational modifications or those which need to be exported from the cell (Pult et al., 2011; Sive et al., 2010). Transporters expressed in this system retain their native properties, showing similar kinetic parameters to those obtained when expressed in other heterologous eukaryotic systems like *S. cerevisiae*. However, the oocyte system is the only one that can be used for electrophysiological transport studies.

Oocytes cells are easy to maintain because they don't require nutrients from the medium and don't need sterile culture conditions. A high proportion of the injected oocytes express the recombinant protein and it is possible to manipulate single cells. Electrophysiological techniques such as patch clamp, two electrode voltage clamp and ion-selective electrodes can also be applied in this system due to the large size of the oocytes (Wagner et al., 2000). On the other hand, the main disadvantages of the system are the high seasonal variation in the oocyte quality, the limited number of injected oocytes that can be used on a single experiment and the short period of time the cells can survive after mRNA injection.

In the first report about *P. falciparum* transporters being functionally expressed in *X. laevis* oocytes, poly(A)$^{+}$-mRNA purified from intraerythrocytic stages of the parasite was injected into the oocytes, resulting in enhanced uptake of D-adenosine, 2'-deoxy-D-glucose and L-lactic acid (Penny et al., 1998). This opened the door for the use of this heterologous system to express parasite membrane proteins. Since then, several *P. falciparum* transporters have been successfully expressed in the *X. laevis* oocyte system, among them the ATPases PfATP4 and PfATP6 (Krishna et al., 2014; Krishna et al., 2001); PfMDR1 (Sanchez et al., 2008a); PfCRT (Martin et al., 2009); PfCHA, a Ca^{+2}/H^{+} antiporter (Rotmann et al., 2010); and the formate-nitrite transporter PfFNT (Marchetti et al., 2015).

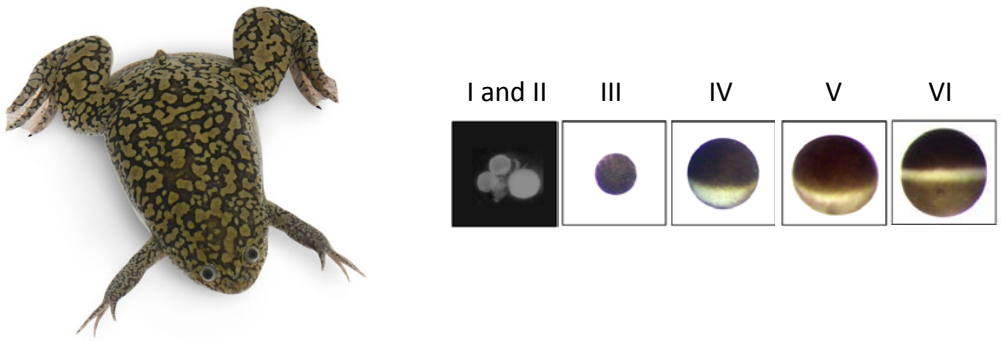


Figure 1.8. Left, the South African clawed toad Xenopus laevis (picture from Nasco's website); right, X. laevis oocytes at different developmental stages.

Thousands of oocytes develop asynchronously in the ovary of *X. laevis* adult females. Stage I oocytes are small (50-300 µm) with transparent cytoplasm, stage II are white and opaque and up to 450 µm in diameter, stage III are still small but already pigmented, stage IV are between 600 and 1000 µm and the animal and vegetal poles start to differentiate, stage V are between 1000 and 1200 µm with clearly delineated hemispheres and stage VI are the largest, with a size between 1200 to 1300 µm showing an unpigmented equatorial band between the vegetal and the animal pole (Dumont, 1972). Oocytes at stages V and VI are the ones used to express heterologous proteins. In the ovary, the oocytes are surrounded by a vitelline envelope and a layer of follicle cells, which has to be removed by collagenase treatment before they can be used experimentally.

1.7. Aim of this study

The aim of this study has been to characterize the function and/or mechanisms of regulation of two *P. falciparum* transporters: PFE0825w (PF3D7_0516500, MAL5P1.165) and PfCRT (PF3D7_0709000, MAL7P1.27).

PFE0825w is a putative organic cation transporter that has been proposed to be a choline carrier (Staines et al., 2010) and one of the putative molecular targets of the compound albitiazolium. It belongs to the Major Facilitator Superfamily (MFS), the largest family of secondary carriers which includes uniporters, antiporters and symporters.

Although progression of albitiazolium to phase III clinical trials was blocked due to its poor bioavailability, further understanding of its mechanism of action could lead to the validation of a new target and the design of new albitiazolium derivatives. Albitiazolium is known to block choline transport inside the parasite; however it is not known for which transporter albitiazolium and choline compete (Wein et al., 2012). Bioinformatic analyses identified the putative organic cation transporter PFE0825w as a possible candidate for this role.

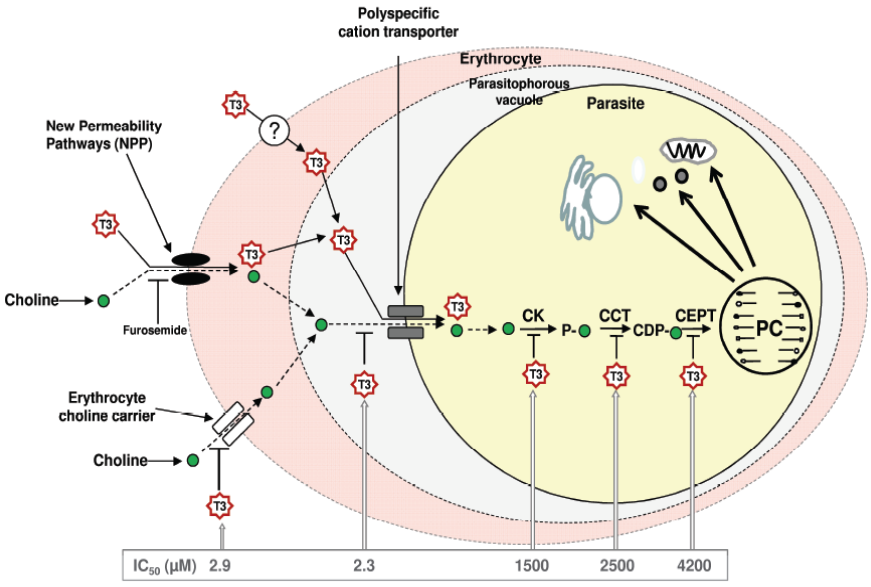


Figure 1.9.Mode of action of the antimalarial drug albitiazolium (Wein et al., 2012).

On the other hand, the gene that codes for this transporter is localized at the C5M3 locus which has been associated with altered CQ responses in the parasite. The F1 progeny

of a GB4x7G8 cross that inherited the C5M3 locus from GB4 showed increased CQ accumulation levels (Sanchez et al., 2011).

One of the aims of this thesis has been to characterize PFE0825w using the *X. laevis* expression system and to confirm or reject its role as antiplasmodial target and resistance mediator.

The second aim of this study has been to evaluate if phosphorylation regulates PfCRT function as a drug carrier and to identify and characterize the kinase(s) which phosphorylate this transporter. Particularly, the role of phosphorylation at positions 33 and 411 in the drug resistance-mediating function of PfCRT has been investigated. The role of the PfCRT T^{416} residue in CQ and QN transport was not assessed since it is essential for PfCRT trafficking to the food vacuole. The substitution of this amino acid by glutamic and aspartic acid only rescues the T^{416}A phenotype partially (Kuhn et al., 2010). Therefore, allelic exchange studies are not suitable to study the role of this residue.

The results might contribute to a deeper understanding of PfCRT regulation, function and role in drug resistance. Furthermore, the identification of the kinase that phosphorylates the residue T^{416} of PfCRT could lead to the identification of a novel antimalarial target. The inhibition of phosphorylation at this particular position could block the trafficking of PfCRT to the food vacuole, impairing the parasite's viability.

Therefore, the characterization of PFE0825w and the mechanisms involved in PfCRT phosphorylation would not only contribute to a better understanding of the parasite's molecular biology, but might also contribute to the identification of new antimalarial targets.

2. Materials and methods

2.1. Materials

2.1.1. Equipment

Equipment	Model	Company
Autoclave	ABT 120-5DM	Kern & Sohn, Balingen, Germany
	2540 EL	Tuttnauer, Breda, The Netherlands
Blot scanner	C-DiGit	Li-cor, Bad Homburg, Germany
Camera	S6X11	Rainbow CCTV, Irvine, CA, USA
Centrifuge	Biofuge fresco	Thermo Fisher Scientific, Dreieich, Germany
	Biofuge pico	Thermo Fisher Scientific, Dreieich, Germany
	J2-MC	Beckman Coulter, Krefeld, Germany
	Megafuge 1.0 R	Heraeus, Hanau, Germany
	Megafuge 2.0 R	Heraeus, Hanau, Germany
	Sorvall RC5B Plus	Thermo Fisher Scientific, Dreieich, Germany
Confocal microscope	LSM510	Zeiss, Jena, Germany
Electrophoresis power supply	Power Pac 300	Bio-Rad, München, Germany
	Power Pac 200	Bio-Rad, München, Germany
	EPS 1001	Amersham (GE Healthcare), München, Germany
	EPS 3501	Amersham (GE Healthcare), München, Germany
Film processor	Hyperprocessor	Amersham (GE Healthcare), München, Germany
Freezer -20°C	LGex 3410 MediLine	Liebherr, Biberach, Germany
Freezer -80°C	HERAfreeze	Thermo Fisher Scientific, Dreieich, Germany
Fridge	LKexv 3910 MediLine	Liebherr, Biberach, Germany
Gel dryer	583	Bio-Rad, München, Germany
Heating block	NeoBlock Mono I	NeoLab, Heidelberg, Germany
Hybridization incubator	Techne HB-1D	Bibby Scientific, Staffordshire, UK
Ice machine		Ziegra, Isernhagen, Germany
Incubator	Heraeus B12/UB12	Thermo Fisher Scientific, Dreieich, Germany

Injector	Nanoject II	Drummond Scientific Company, Broomall, PA, USA
Light optical microscope	Axio Lab.A1	Zeiss, Jena, Germany
Liquid nitrogen tank	MVE Cryosystem 6000	Thermo Fisher Scientific, Dreieich, Germany
	LS 6000	Taylor-Wharton, Husum, Germany
	RS Series	Taylor-Wharton, Husum, Germany
Liquid scintillation counter	LS6000IC	Beckman Coulter, Krefeld, Germany
Magnetic sorter	VarioMACS	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic stirrer	RCT	IKA, Staufen, Germany
	COMBIMAG RCH	IKA, Staufen, Germany
	HR 3001	Heidolph, Schwabach, Germany
Microwave oven	R940/94ST	Sharp, Hamburg, Germany
MiliQ water system	Purist ultrapure	Rephile, Germany
Particle counter	Z1	Beckman Coulter, Krefeld, Germany
pH meter	pH 7110	WTW, Weilheim, Germany
Pipetman	P10	Gilson, Limburg an der Lahn, Germany
	P20	Gilson, Limburg an der Lahn, Germany
	P1000	Gilson, Limburg an der Lahn, Germany
Pipetus	Forty\Standard	Hirschmann, Eberstadt, Germany
Plate reader	FLUOstar OPTIMA	BMG Labtech, Ortenberg, Germany
PyroMark	Q96 ID	QIAGEN, Hilden, Germany
Semi-dry transfer cell	Trans-blot SD	Bio-Rad, München, Germany
Shaker	KS 501 digital	IKA, Staufen, Germany
Shaker incubator	Innova 4300	Eppendorf (New Brunswick), Wesseling-Berzdorf, Germany
	Innova 4000	Eppendorf (New Brunswick), Wesseling-Berzdorf, Germany
Sonicator	Sonoplus HD 2070	Bandelin, Berlin, Germany
Spectrophotometer	UVIKON 923	Kontron instruments, Munich, Germany
Sterile work bench	Herasafe	Thermo Fisher Scientific, Dreieich, Germany
	SterilGard Class II	The Baker company, Sanford, ME, USA
Thermocycler	Labcycler	Sensoquest, Göttingen, Germany

UV chamber	GS Gene linker	Bio-Rad, München, Germany
UV table	TFX-35M	Vilber Lourmat, Eberhardzell, Germany
Vortex	Genie 2	Scientific Industries, Bohemia, NY, USA
Vacuum Workstation	PyroMark Q96	QIAGEN, Hilden, Germany
Waterbath	7A	Julabo, Seelbach, Germany
	5B	Julabo, Seelbach, Germany

2.1.2. Disposables

Disposable	Company
96 well cell culture plates	Greiner bio one, Frickenhausen, Germany
Aluminium foil	Carl Roth, Karlsruhe, Germany
Cellstar tubes	Greiner bio one, Frickenhausen, Germany
Centrifuge bottles Nalgene 500 ml	Thermo Fisher Scientific, Dreieich, Germany
Clingfilm Saran	Dow, Schwalbach, Germany
Cuvettes Gene Pulser	Bio-Rad, München, Germany
Filters Millex GS (0,2µm)	Merck Millipore, Darmstadt, Germany
Filter systems 500 ml	Corning, Kaiserslautern, Germany
Gloves TouchNTuff	Ansell, München, Germany
MACS CS column	Miltenyi Biotec, Bergisch Gladbach, Germany
Micro tubes 1.5 ml	Saarstedt, Nümbrecht, Germany
Parafilm	Bemis, Londonderry, UK
Petri dishes (10 ml diameter)	Greiner bio one, Frickenhausen, Germany
Petri dishes (25 ml diameter)	Greiner bio one, Frickenhausen, Germany
Pipette tipps	Corning, Kaiserslautern, Germany
Plastic pipettes (1 ml; 2 ml; 5 ml; 10 ml; 25 ml)	Corning, Kaiserslautern, Germany
Precision wipes (11x21cm)	Kimberly Clark, Mainz, Germany
Polypropylene tubes (14 ml)	Greiner bio one, Frickenhausen, Germany
PyroMark Q96 Plate Low	QIAGEN, Hilden, Germany
Scalpel 11, disposable	Feather, Osaka, Japan

Strip tubes & domed caps	BioMedical Instruments, Zoellnitz, Germany
Stiches Safil 4/0 45 cm	B|Braun, Melsungen, Germany
Transfer pipettes	Sarstedt, Nümbrecht, Germany
Vials Mini PolyQ	Beckman Coulter, Krefeld, Germany
XAR biomax films	Kodak, Stuttgart, Germany

2.1.3. Kits

Kit	Company
BM chemiluminescence blotting substrate POD	Roche, Mannheim, Germany
CloneJET PCR Cloning kit	Fermentas - Thermo Fischer Scientific, Dreieich, Germany
DNeasy Blood & Tissue kit	QIAGEN, Hilden, Germany
FastTrack MAG mRNA isolation kit	Ambion - Thermo Fisher Scientific, Dreieich, Germany
Gel extraction kit	QIAGEN, Hilden, Germany
High pure plasmid miniprep kit	Roche, Mannheim, Germany
In-Fusion HD Cloning kit	Clontech - Takara Bio Europe, Saint-Germain-en-Laye, France
In vitro RNA transcription kit (mMessage mMachine SP6)	Ambion - Thermo Fisher Scientific, Dreieich, Germany
Matchmaker Gold Yeast Two-Hybrid System	Clontech - Takara Bio Europe, Saint- Germain-en-Laye, France
PCR purification kit	QIAGEN, Hilden, Germany
Pierce co-immunoprecipitation kit	Thermo Fisher Scientific, Dreieich, Germany
Plasmid maxiprep kit	QIAGEN, Hilden, Germany
SuperScript III First Strand Synthesis System for RT-PCR	Invitrogen - Thermo Fisher Scientific, Dreieich, Germany

2.1.4. Chemicals

2.1.4.1. Non-radioactive chemicals

All non-radioactive chemicals used during this study were purchased from one of the following companies: Boehringer Ingelheim, Carl Roth, General Electric Company including the trademarks it owns (GE Healthcare and Amersham), Honeywell, ICN Biomedicals, Merk Millipore including the trademarks it owns (Calbiochem, Novagen), MP Biomedicals, Roche, Sigma-Aldrich including the trademarks it owns (Fluka) and Thermo Fisher Scientific including the trademarks it owns (Gibco, Invitrogen, Molecular Probes).

2.1.4.2. Radioactive chemicals

Chemical	Reactivity	Concentration	Company
[γ-^{32}P]-dATP	6000 Ci/mmol	10 mCi/ml	PerkinElmer, Baesweiler, Germany
[^{14}C]-albitiazolium		100 mM	Sanofi, Chilly-Mazarin, France
[^{3}H]-chloroquine	25 Ci/mmol	1 mCi/ml	GE Healthcare, München, Germany
[^{3}H]-choline	85.5 Ci/mmol	1 mCi/ml	PerkinElmer, Baesweiler, Germany
[^{3}H]-MPP	80 Ci/mmol	1 mCi/ml	Biotrend, Köln, Germany
[^{3}H]-quinine	20 Ci/mmol	1 mCi/ml	Biotrend, Köln, Germany
[^{14}C]-TEA	55 mCi/mmol	0.1 mCi/ml	Biotrend, Köln, Germany

2.1.5. Biological materials

2.1.5.1. Size Markers

Marker	Company
GeneRuler 1 Kb plus DNA ladder	Ambion - Thermo Fisher Scientific, Dreieich, Germany
PageRuler Plus Prestained protein ladder	Ambion - Thermo Fisher Scientific, Dreieich,Germany

2.1.5.2. Antibodies

Antibody	Source	Company
Anti-α-tubulin monoclonal	Mouse	Sigma Aldrich, München, Germany
Anti-BIP polyclonal	Rabbit	Provided by J. Przyborski (Pesce et al., 2008)
Anti-GFP monoclonal	Mouse	Roche, Mannheim, Germany
Anti-guinea pig-POD monoclonal	Donkey	Jackson ImmunoResearch, Suffolk, UK
Anti-HA tag monoclonal	Mouse	Roche, Mannheim, Germany
Anti-His tag monoclonal	Mouse	Merck Millipore, Darmstadt, Germany
Anti-InsP_3R-II polyclonal	Rabbit	Sigma Aldrich, München, Germany
Anti-mouse-POD monoclonal	Donkey	Jackson ImmunoResearch, Suffolk, UK
Anti-PfCRT polyclonal	Guinea pig	Eurogentec, Köln, Germany
Anti-rabbit-POD monoclonal	Goat	Jackson ImmunoResearch, Suffolk, UK

2.1.5.3. Enzymes

Enzyme	Company
Calf intestinal alkaline phosphatase	New England BioLabs, Frankfurt am Main, Germany
Collagenase Type IA	Sigma Aldrich, München, Germany
EuroTaq DNA polymerase	BioCat, Heidelberg, Germany
Phusion Polymerase	Finnzymes - Thermo Fisher Scientific, Dreieich, Germany
Restriction enzymes	New England BioLabs, Frankfurt am Main, Germany
Shrimp Alkaline Phosphatase	Promega, Mannheim, Germany
T4 DNA Ligase	Thermo Fisher Scientific, Dreieich, Germany

2.1.5.4. Plasmids

All the plasmids used in this study are described in Appendix I.

2.1.5.5. Organisms

Organism	Strain	Origin
E. coli	PMC 103	Provided by Prof. Cowman (Doherty et al., 1993)
E. coli	XL10 Gold	Agilent Technologies, Böblingen, Germany
E. coli	BL21(DE3)	Novagen- Merck Millipore, Darmstadt, Germany
E. coli	BL21-CodonPlus- RIL	Agilent Technologies, Böblingen, Germany
S. cerevisiae	AH109	Takara, Saint-Germain-en-Laye, France
P. falciparum	3D7	The Netherlands (Walliker et al., 1987)
P. falciparum	7G8	Brazil (Hadley et al., 1987)
P. falciparum	Dd2	Indochina (Guinet et al., 1996)
P. falciparum	GB4	Ghana (Sullivan et al., 2003)
P. falciparum	HB3	Honduras (Bhasin and Trager, 1984)
X. laevis		NASCO, Fort Atkinson, WI, USA

2.1.5.6. Oligonucleotides

All oligonucleotides used in this study were purchased from Thermo Fisher Scientific or Eurofins.

- *Vector oligonucleotides for colony PCR and sequencing:*

Nº	Name	Sequence
1	pJET-for	CGACTCACTATAGGGAGAGCGGC
2	pJET-rev	AAGAACATCGATTTTCCATGGCAG
3	pSP64T-for	AGAATACAAGCTTGCTTGTTC
4	pSP64T-rev	GTAAGTTGGTATTATGTAG
5	pL6-guide-for	GTAACCAAAATGCATAATTTTTCC
6	pL6-guide-rev	TAGGAAATAATAAAAAAGCACC
7	pL6-HA-glmS-3'-for	ATCACATGATCTTCCAAAAAACATG
8	pL6-HA-glmS-3'-rev	TAAACCAATAGATAAAATTTGTAGAG
9	pL6- HA-glmS-5'-for	ATTTAACTATATACTATGGAATAC
10	pL6- HA-glmS-5'rev	TATTGAGAAAATAAGAACAAGAC
11	pARL-for	CTATAATATCCGTTAATAATAAATACACGCAGTC

12	pARL-rev	CACAACATACACATTTTTACAG
13	Matchmaker 3' AD	GTGAACTTGCGGGGTTTTTCAGTATCTACGATT
14	Matchmaker 5' AD	CTATTCGATGATGAAGATACCCCACCAAACC

- *PFE0825w oligonucleotides:*

In blue, enzyme restriction sites.

Nº	Name	Sequence
15	PFE0825w-for	ATGGAAGTAACATCAACCTTATTAG
16	PFE0825w-rev	TTATAAAATCGACTTAATACTGG
17	PFE0825w-ga-XhoI-for	CCGCTCGAGATGGAAGTTACTTCTACCTTG
18	PFE0825w-ga-NcoI-rev	CATGCCATGGTTAGTGGTGGTGGTGGTGGTGCAAG
19	PFE0825w-ga-var1-XhoI-for	CCGCTCGAGATTATGGTTTGTGAATCCACC
20	PFE0825w-var2-XhoI-for	CCGCTCGAGGAAATGTTTATGTATTTATATATTTT
21	PFE0825w-var2-rev	CAGGTGGATTCACAAACCATGATCATTAGGTACATTAAGC
22	PFE0825w-ga-(-his)-NcoI-rev	CATGCCATGGTTACAAGATGGATTTGATGGAAGAGAAC
23	PFE0825w-exon5-for	GCAAACATTTCAAGCTTCCT
24	PFE0825w-exon5-rev	CGAAATTTGTTTTTAAGCACAC

- *PfCRT oligonucleotides:*

In green, homology regions for *In Fusion* cloning,* phosphorothioate-modified bases.

Nº	Name	Sequence
25	PfCRT-(-120)-In fusion-for	ATGGCCCCTTTCCGCAAATATTTTAAAATCGACATTCCG
26	PfCRT-(780)-In fusion-rev	TTTTTACAAAATGCTACTGAACAGGCATCTAACATGG
27	PfCRT-(-120)-for	A*A*ATATTTTAAAATCGACATTCCG
28	PfCRT-(780)-rev	A*C*TGAACAGGCATCTAACATGG
29	PfCRT-S33A-for	CTTAACAGATGGAGCACGTTTAGGTGG
30	PfCRT-S33A-rev	CCACCTAAACGTGCTCCATCTGTTAAG
31	PfCRT-guide3-for	TAAGTATATAATATTTAAACGTGAGCCATCTGTTAGTTTTAGAGCTAGAA
32	PfCRT-guide3-rev	TTCTAGCTCTAAAACTAACAGATGGCTCACGTTTAAATATTATATACTTA
33	Pfcrt-(900)-rev	TTTTTACAAAATGCTTAAAATAGTATACTTACCTATATC
34	PfCRT(-160)-Bio	CATTGTCTTCCACATATATGAC
35	PfCRT-S33A-Pyro-rev-2	AATAAGTTTAAACACATGAGCAC
36	PfCRT-S33A-Pyro-seq	CCAAGACAAGAACCTCCACCTAAAC

- *PF11_0488 oligonucleotides:*

In blue, enzyme restriction sites; in green, homology regions for *In Fusion* cloning; in red, shield mutations.

Nº	Name	Sequence
37	PF11_0488-(3529)-SpeI-for	GGACTAGTGAAATGTATGCAGCCAAAATTC
38	PF11_0488-BssHII-rev	TTGGCGCGCCTGGTATATTAAAGTAGTTAAAAATTGG
39	PF11_0488-shld1-BssHII-rev	TTGGCGCGCCTGGTATATTAAAGTAGTTAAAAATTGGATAGCTCAATG
40	PF11_0488-shld2-for	GACCTTTCCAATTTAATTATTTAGAAAAATGTTCAAAAG
41	PF11_0488-shld2-rev	CTTTTGAACATTTTTCTAAATAATTAAATTGGAAAGGTC
42	PF11_0488-3'UTR-NarI-for	GAGGCGCCAGAAATTATATATATATATCATTAAATATTTTG
43	PF11_0488-3'UTR-AlfII-rev	AGCTTAAGGAATTTTAAAGAATTCATTGTTCGCATTTG
44	PF11_0488-guide1-for	TAAGTATATAATATTGTTAAAAATTGGATAGCTTAGTTTTAGAGCTAGAA
45	PF11_0488-guide1-rev	TTCTAGCTCTAAAACTAAGCTATCCAATTTTTAACAATATTATATACTTA
46	PF11_0488-guide2-for	TAAGTATATAATATTATTCTTTTGAACATTTTTCAGTTTTAGAGCTAGAA
47	PF11_0488-guide2-rev	TTCTAGCTCTAAACTGAAAATGTTCAAAAGAATAATATTATATACTTA
48	PF11_0488-(-62)-3'UTR-rev	GTATTACAATGAGTTATAAGAAATAATCC
49	GlmS-XmaI-rev	TCCCCCCGGGAGATCATGTGATTTCTCTTTGTTC
50	PF11_0488-(3328)-NheI-for	CTAGCTAGCATGAAATTAAATTTGGATAAAAAAAGAGC
51	PF11_0488-XhoI-rev	CCGCTCGAGTGGTATATTAAAGTAGTTAAAAATTGG

2.1.6. Buffers, media and solutions

Buffer/media/solution	Composition
Anesthetic solution	0.1% (w/v) ethyl 2-aminobenzoate methanesulfonate salt in tap water
Annealing buffer (pyrosequencing)	20 mM Tris 2 mM magnesium acetate Set pH to 7.6 with HCl and autoclave
Binding buffer (pyrosequencing)	10 mM Tris 2 M NaCl 1 mM EDTA Set pH to 7.6 with HCl and autoclave Add 0.1% Tween 20

Blocking solution	5% (w/v) skimmed milk in PBS
Collagenase solution	0.1% (w/v) of collagenase D 0.5% (w/v) of BSA 9 mM Na_2HPO_4 in OR2 buffer
Complete RPMI/HEPES medium	10% human serum 0.2 mM hypoxanthine 0.002% (w/v) gentamicin in RPMI/HEPES medium
Coomassie destaining solution	20% methanol 7% acetic acid
Coomassie staining solution	50% methanol 10% acetic acid 0.5% Coomassie Blue R-250
Cytomix	25 mM HEPES pH 7.6 120 mM KCl 0.15 mM $CaCl_2$ 2 mM EGTA 5 mM $MgCl_2$ 10 mM K_2HPO_4/KH_2PO_4
Denaturation solution (pyrosequencing)	0.2 M NaOH
Dialysis buffer	20 mM Tris-HCl 20 mM $MgCl_2$ 2 mM $MnCl_2$ 10% glycerol Protease inhibitors
DNA loading buffer (6x)	60% glycerol 60 mM EDTA 0.25% Bromophenol blue

Freezing solution	6.2 M glycerol 0.14 M sodium lactate 0.5 mM KCl Set pH to 7.2 with $NaHCO_3$ pH 9 Sterilize by filtration
Kinase assay buffer	20 mM Tris-HCl 20 mM $MgCl_2$ 2 mM $MnCl_2$ 2 mM DTT 100 µM ATP 50 mM β-GP 0.5% phosphatase inhibitors cocktail (Sigma Aldrich, München, Germany) Add fresh protease inhibitors before use
LiAc (10x)	1 M lithium acetate Adjust to pH 7.5 with glacial acetic acid Sterilize by filtration
LB	1% (w/v) tryptone 0.5% (w/v) yeast extract 0.5% (w/v) NaCl Autoclave
LB Agar	1% (w/v) tryptone 0.5% (w/v) yeast extract 0.5% (w/v) NaCl 1.5% (w/v) agar Autoclave and pour into petri dishes (~25 ml/100 mm plate)
Lysis buffer for IC_{50}	20 mM Tris-HCl pH 7.4 5 mM EDTA 0.008% (w/v) saponin 0.08% (w/v) triton X-100

Lysis buffer for *S. cerevisiae* DNA isolation	10 mM Tris-HCl pH 8.0 1 mM EDTA 100 mM NaCl 1% (w/v) SDS 2% (w/v) triton-X
Low salt buffer	10 mM $NaPO_4$ Set pH to 7.0 with NaOH and autoclave
MACS buffer	2 mM EDTA 1 x PBS Autoclave Add 0.5% (w/v) BSA prior to use
ND96	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 1.8 mM $CaCl_2$ 10 mM HEPES Set pH to 7.5 with NaOH and autoclave Add 20 U/ml of penicillin/streptomycin
NETT buffer	10 mM Na_3PO_4 pH 7.0 150 mM NaCl 1 mM EDTA 0.1% NP40
NETT I buffer	10 mM Na_3PO_4pH 7.0 250 mM NaCl 1 mM EDTA 0.1% NP40
NETT II buffer	10 mM Na_3PO_4 pH 7.0 350 mM of NaCl 1 mM EDTA 0.1% NP40

NZY+ Broth	1% (w/v) NZ amine (casein hydrolysate) 0.5% (w/v) yeast extract 0.5% (w/v) NaCl Set pH to 7.5 with NaOH and autoclave Add the following filter-sterilized supplements prior to use: 12.5 mM $MgCl_2$ 12.5 mM $MgSO_4$ 0.4% (w/v) glucose
OR2 buffer	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 10 mM HEPES Set pH to 7.5 with NaOH and autoclave
Protease inhibitors (PI)	0.002% (w/v) leupeptin 0.005% (w/v) aprotinin 100 µM PMSF
Protein loading buffer (2x)	3% (w/v) SDS 250 mM Tris pH 6.8 20% glycerol 0.1% Bromophenol blue
Protein lysis buffer for *P. falciparum*	0.07% (w/v) saponin in PBS Protease inhibitors
Protein lysis buffer for *E. coli*	1x PBS 5% (w/v) glycerol 0.1% triton X-100 1 mM EDTA 1 mM DTT Add fresh before use: Protease inhibitors 0.01% (w/v) lysozyme
Protein lysis buffer for *S. cerevisiae*	40 mM Tris-HCl pH 6.8 0.1 mM EDTA 5% (w/v) SDS 8 M urea Add fresh before use: Protease inhibitors 1 % β-mercaptoethanol

Protein purification wash buffer (for GST-tagged proteins)	25 mM Tris-HCl pH 7.0 75 mM NaCl 5% glycerol 1 mM DTT Protease inhibitors
Protein purification wash buffer (for His-tagged proteins)	50 mM Na_3PO_4 300 mM NaCl 150 mM imidazole Adjust to pH 7.4 with NaOH Add protease inhibitors
Protein elution buffer (for GST-tagged proteins)	50 mM Tris pH 7.0 75 mM NaCl 5% glycerol 1 mM DTT 10 mM reduced glutathione Add protease inhibitors
Protein elution buffer (for His-tagged proteins)	50 mM Na_3PO_4 300 mM NaCl 400 mM imidazole Adjust to pH 7.4 with NaOH Add protease inhibitors
Ringer solution	10 mM HEPES 122.5 mM NaCl 5.4 mM KCl 1.2 mM $CaCl_2$ 0.8 mM $MgCl_2$ 1 mM NaH_2PO_4 11 mM glucose Adjust to pH 7.4 with NaOH Sterilize by filtration
RIPA buffer	10 mM Na_3PO_4 pH 7.0 150 mM NaCl 1 mM EDTA 1% NP40 1% DOC 0.1% SDS
RNA running buffer (20x)	400 mM MOPS 40 mM $C_2H_3NaO_2$ 5 mM of EDTA

SB	3.5% (w/v) tryptone 2% (w/v) yeast extract 0.5% (w/v) NaCl 2 mM NaOH Autoclave
SD medium	0.67% (w/v) yeast nitrogen base Ø amino acids 2% (w/v) agar (for plates only) Add the recommended amount of the appropriate OD supplement according to the manufacturer's instructions. Set pH to 5.8 with NaOH and autoclave Add 2% (w/v) glucose
SDS-PAGE running buffer	25 mM Tris 250 mM glycine 0.1% (w/v) SDS
SDS-PAGE transfer buffer	39 mM Tris 48 mM glycine 0.038% (w/v) SDS
SOB medium	2% (w/v) tryptone 0.5% (w/v) yeast extract 0.05% (w/v) NaCl 0.5% (w/v) MgSO4*7H2O Autoclave
SOC medium	SOB + 20 mM glucose
Stripping buffer	1x PBS 2% SDS 100 mM β-mercaptoethanol
Thawing solution I	12% (w/v) NaCl Autoclave

Thawing solution II	1.6% (w/v) NaCl Autoclave
Thawing solution III	0.9% (w/v) NaCl 0.2% (w/v) glucose Sterilize by filtration
TB Buffer	10 mM PIPES 15 mM $CaCl_2$ 250 mM KCl Set pH to 6.7 with KOH Add 55 mM $MnCl_2$ Sterilize by filtration
TE buffer	10 mM Tris-HCl pH 7.5 1 mM EDTA Sterilize by filtration
Transfection medium	5% (w/v) albumax I 5% serum 0.2 mM hypoxanthine 0.002% gentamicin in RPMI/HEPES medium
Uptake buffer oocytes (pH 7.4)	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 1 mM $CaCl_2$ 10 mM HEPES Set pH to 7.4 with NaOH and autoclave
Uptake buffer oocytes (pH 6.0)	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 1 mM $CaCl_2$ 10 mM MES Set pH to 6.0 with NaOH and autoclave
Uptake buffer oocytes (pH 5.0)	96 mM NaCl 2 mM KCl 1 mM $MgCl_2$ 1 mM $CaCl_2$ 10 mM HOMOPIPES Set pH to 5.0 with NaOH and autoclave

Wash buffer (pyrosequencing)	10 mM Tris Set pH to 7.6 with HCl and autoclave
YPDA medium	2% (w/v) peptone 1% (w/v) yeast extract 2% (w/v) agar (for plates only) 0.003% (w/v) of adenine hemisulfate Autoclave Add 2% (w/v) glucose

2.1.7. Computer software and databases

Program	**Company**
Bioedit	Ibis Biosciences, Carlsbad, CA, USA
EndNote	Thomson Reuters, Philadelphia, PA, USA
SigmaPlot 11.0	Systat, San Jose, CA, USA
SnapGene Viewer	GSL Biotech, Chicago, IL, USA
LSM imaging software	Zeiss, Jena, Germany
FIJI	(Schindelin et al., 2012)
Databases and online bioinformatic tools	
PlasmoDB	
Prosite	
Sequence manipulation suite	
PhosphoMotif Finder	
TMHMM server v. 2.0	

2.2. Methods

2.2.1. Methods in molecular biology

2.2.1.1. Genomic DNA purification from *P. falciparum*

The genomic DNA was extracted and purified from *P. falciparum* cultures using the DNeasy Blood & Tissue Kit from QIAGEN. The cultures (14 ml, parasitemia 3-5% trophozoites, 3.6% hematocrit) were centrifuged at 900 x g for 2 min, the supernatant was discarded and the pellet was resuspended in 10 ml of cold PBS. The samples were lysed with saponin (final

concentration 0.1% w/v) for 5 min on ice. After a centrifugation of 10 min at 2600 x g, the supernatant was discarded and the pellet was washed twice with cold PBS. The gDNA extraction was performed according to the manufacturer's instructions. Briefly, the samples were lysed with proteinase K and lysis buffer and purified using a silica-based membrane that absorbs the DNA in presence of high concentrations of chaotropic salts. The samples were loaded onto the DNeasy spin column and centrifuged. After two washing steps, the genomic DNA was eluted in 100 µl of ddH_2O and stored at -20°C.

2.2.1.2. Polymerase chain reaction

The amplification of the DNA fragments used for cloning was done using either the Phusion polymerase or a mix of *Taq:Pfx* (4.5:1) polymerases. The reactions were set as follows:

Phusion	*Taq:Pfx*
4 µl buffer 5x	5 µl buffer 10x
2 µl template (100 ng)	2 µl template (100 ng)
2 µl dNTPs (2 mM)	5 µl dNTPs (2 mM)
0.5 µl primer for (50 µM)	2.5 µl $MgCl_2$
0.5 µl primer rev (50 µM)	1 µl primer for (50 µM)
0.2 µl Phusion	1 µl primer rev (50 µM)
10.8 µl of ddH_2O	0.5 µl Taq:Pfx
20 µl total volume	33 µl of ddH2O
	50 µl total volume

The program used in the thermocycler was the following:

95 °C	10 min	
95 °C	45 s	30 cycles
X °C	45 s	
68 °C	X min	
68 °C	10 min	
4 °C	∞	

2.2.1.3. Agarose gel electrophoresis

- DNA

The size of all DNA fragments used during cloning and the final vectors used on this study was checked by agarose gel electrophoresis. The agarose was dissolved to a final concentration of 0.8% or 2% (w/v) on TAE buffer. The DNA was stained using ethidium bromide. As a marker, the GeneRuler 1 Kb plus DNA ladder was loaded in parallel with the

samples. The 60 ml gels were run at 90 V and the 140 ml gels at 140 V during approximately 30-45 min. The DNA fragments were visualized using a UV table and the images were captured with a digital camera.

- RNA

The quality of all RNA samples was checked by agarose gel electrophoresis. The agarose was dissolved in 30 ml of ddH_2O plus 2 ml of 20x RNA running buffer to a final concentration of 0.7%. The solution was allowed to cool down to 55°C and then 7.9 ml of formaldehyde were added. After addition of 0.5 µl of ethidium bromide, the solution was allowed to cool down until it was solidified. After the addition of RNA loading buffer (Ambion - Thermo Fisher Scientific), the samples were heated at 65°C during 3 min and loaded into the gel. The gel was run at 60 V during 60 min. The RNA samples were visualized using a UV table and the images were captured with a digital camera.

2.2.1.4. DNA restriction digestion

The DNA digestion reactions of plasmids and PCR products were set up as follows:

Control digestions	Digestion of vectors and inserts for cloning
1 µl NEB buffer	5 µl NEB buffer
0.5 µl of each enzyme	1 µl of each enzyme
1 µg DNA	20 µg DNA
x µl ddH_2O	x µl ddH_2O
10 µl total volume	50 µl total volume

The control digestions were incubated for 90 min and the digestions of vectors and inserts for cloning for a minimum of 2 hours at the temperature for each enzyme recommended by NEB.

2.2.1.5. DNA gel extraction

The extraction and purification of DNA fragments from agarose gels was performed using the QIAquick Gel Extraction Kit from QIAgen according to the manufacturer's instructions. Briefly, the DNA fragments were excised from the agarose gel and dissolved in QG buffer which provides optimal pH and salt concentration for binding of DNA to a silica membrane. The samples were loaded onto the QIAquick spin column and centrifuged. After two washing steps, the DNA fragments were eluted in 30-50 µl of ddH_2O and stored at -20°C.

2.2.1.6. Dephosphorylation of DNA ends

All the vectors used for cloning were dephosphorylated prior to their use on ligation reactions. The reactions were set as follows:

20 µg of vector
1 µl of SAP
1 µl of SAP buffer 10x
x µl of ddH_2O

10 µl total volume

The reaction was incubated for 30 min at 37°C and then the vector was purified using the QIAGEN PCR Purification kit and eluted in 30 µl of ddH_2O.

2.2.1.7. Ligation of DNA fragments

- *In Fusion*

The *In Fusion* cloning technology was used to introduce the guide sequences into the pL6 plasmids. The pL6 plasmid of interest was digested with the enzyme BtgZI at 60°C for 1 h, dephosphorylated and purified with the QIAquick gel extraction kit. The primers containing the guide sequences and the sequences for homology recombination were resuspended at 100 µM and 5 µl of each primer (for and rev) were mixed together with 1.1 µl of NEB buffer #2. The mix was heated for 5 min at 94°C, was allowed to cool down to 25°C and was kept on ice. The reaction was set as follows:

0.5 µl vector (200 ng)
3.5 µl hybridized primers (1/10 dilution)
1 µl *In Fusion* enzyme mix

The reaction was incubated for 15 min at 50°C and was kept on ice until its transformation into XL-10 Gold cells.

- T4 DNA ligation

The DNA fragments that were ligated using the T4 DNA ligase were previously digested with the adequate restriction enzymes and were purified using the QIAquick gel extraction kit. The ligation reactions were set as follows:

1 µg vector
3-7 µg insert
1 µl T4 DNA ligase
1 µl T4 DNA ligase buffer 10x
x µl ddH_2O

10 µl total volume

The reaction was incubated at RT during minimum 30 min. If the transformation was performed on the following day, then the reaction was incubated over-night at 16°C.

- Ligation into pJET1.2/blunt

As an intermediate cloning step, many of the inserts used on this study were cloned into the pJET1.2/blunt plasmid. The CloneJET PCR Cloning Kit (Fermentas - Thermo Fischer Scientific) was used for this purpose. The reaction was set as follows:

5 µl 2X reaction buffer
0.5 µl DNA blunting enzyme
3.5 µl insert (100 ng)

The reaction was incubated at 70°C for 5 min and chilled on ice. Then the following reagents were added:

0.5 µl pJET1.2/blunt cloning vector (50 ng/µl)
0.5 µl T4 DNA ligase

The ligation mixture was incubated at RT for 30 min. The reaction was used directly for transformation in *E. coli* PMC 103 electrocompetent cells.

2.2.1.8. Plasmid DNA isolation from *E. coli*

- Small scale (miniprep)

The DNA plasmid isolation from bacteria was performed using the High pure plasmid miniprep Kit from Roche according to the manufacturer's instructions. The purification principle is based in alkaline lysis followed by DNA absorption in a glass fiber fleece immobilized in a plastic filter tube. Briefly, bacterial over-night cultures (10 ml) were centrifuged for 5 min at 1100 x g. The pellet was resuspended in suspension buffer and lysed with lysis buffer for 5 min at RT. The samples were incubated on ice for 5 min after addition of chilled binding buffer and centrifuged for 10 min at 17000 x g. The supernatant was

transferred onto a High Pure filter tube and centrifuged for 1 min at 17000 x g. After two washing steps, the plasmids were eluted in 50 µl of ddH_2O and stored at -20°C.

- Large scale (maxi prep)

In order to isolate higher amounts of plasmid, for example for transfection, the QIAGEN Plasmid Maxi kit was used according to the manufacturer's instructions. The protocol is based on alkaline lysis followed by DNA binding to an anion-exchange resin. Briefly, bacterial over-night cultures (400 ml of SB medium) were centrifuged at 4°C for 15 min at 20000 x g. Each pellet was resuspended in resuspension buffer and lysed with the lysis buffer for 5 min at RT. Next, the samples were incubated for 20 min on ice with neutralization buffer, centrifuged at 4°C for 30 min at 15000 x g and the supernatant was transferred to a column previously equilibrated. After washing, the DNA was eluted in 15 ml of elution buffer, precipitated with isopropanol and centrifuged at 4°C for 30 min at 15000 x g. The pellet was washed with ethanol and centrifuged at 4°C for 20 min at 15000 x g. The DNA pellet was air-dried, resuspended in 300 µl of ddH_2O and stored at -20°C.

2.2.1.9. Plasmid DNA isolation from *S. cerevisiae*

One single colony was inoculated in 10 ml of the appropriate YPDA or SD medium and was incubated over-night at 30°C with shaking. Next, the culture was centrifuged at 1600 x g for 5 min, the supernatant was discarded and the pellet was resuspended in 200 µl of lysis buffer for *S. cerevisiae* DNA isolation. The suspension was added to a new 1.5 ml tube containing 300 mg of glass beads and 200 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1). The tube was vortexed for 5 min and centrifuged at 4°C for 5 min at 17000 x g. The upper phase (150 µl) was transferred to a new tube and was mixed with 150 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1). The sample was centrifuged again under the same conditions and the upper phase was precipitated with sodium acetate / ethanol (1/10 volumes of 2.5 M sodium acetate and 2.5 volumes of 100% ethanol). After the ethanol precipitation and one wash with ethanol 70%, the pellet was air-dried and resuspended in 10 µl of ddH_2O.

2.2.1.10. Sequencing of DNA

The sequencing of the DNA samples was performed by GATC Biotech (Konstanz, Germany). The samples were prepared as follows:

Plasmids: 20 µl, 30-100 ng/µl
PCR products: 20 µl, 10-50 ng/µl
Primers: 20 µl, 10 pmol/µl

2.2.1.11. RNA isolation

Two big plates (35 ml, 3-5% parasitemia in trophozoites, 3.6% hematocrit) were lysed with saponin 0.1% (w/v) in cold PBS. The pellet after centrifugation (3000 x g, 2 min without brake) was resuspended in 1 ml of TRIzol® LS reagent (Ambion - Thermo Fisher Scientific). The homogenized sample was incubated for 10 min at RT and 200 µl of chloroform were added. The tube was shaken vigorously by hand and incubated 10 min at RT. The sample was centrifuged at 4°C for 30 min at 17000 x g. The aqueous phase of the sample, containing the RNA, was pipetted into a new tube and 500 µl of 100% isopropanol were added. The sample was incubated for 10 min at RT and then centrifuged at 4°C for 10 min at 17000 x g. The supernatant was discarded; the pellet was washed with 1 ml of 70% (v/v) ethanol and centrifuged at 4°C for 5 min at 17000 x g. The supernatant was discarded; the pellet was air-dried for 10 min and then resuspended in 30 µl of ddH_2O.

2.2.1.12. Determination of the DNA/RNA concentration

The DNA/RNA concentration of the samples was measured using the spectrophotometer UVIKON 923. The OD spectrum from 230 to 300 nm of a 1/100 dilution of the sample (or a lower dilution if it was necessary) was measured against a blank (ddH_2O). The DNA/RNA concentration of the sample was calculated as follows:

1 O.D. at 260 nm for double-stranded DNA = 50 ng/µl
1 O.D. at 260 nm for RNA molecules = 40 ng/µl of RNA

2.2.1.13. cDNA synthesis

The cDNA synthesis from RNA was performed using the SuperScript III First Strand Synthesis kit from Invitrogen. The reaction was set up as follows:

5 µg RNA
1 µl 50 µM oligo $(dT)_{20}$
1 µl 10 mM dNTP mix
x µl ddH_2O

---

10 µl final volume

The mix was incubated at 65°C for 5 min and then placed on ice for 5 min. Next, the following reagents were added:

2 µl 10X RT buffer
4 µl 25 mM $MgCl_2$
2 µl 0.1 M DTT
1 µl RNaseOUT (40 U/µl)
1 µl SuperScript III RT (200 U/µl)

20 µl final volume

The reaction was incubated at 50°C for 50 min and terminated at 85°C for 5 min. After it was chilled on ice, 1 µl of RNase H was added and the reaction was incubated for 20 min at 37°C. The cDNA was stored at -20°C until it was used as PCR template.

2.2.1.14. *In vitro* synthesis of RNA

The *in vitro* synthesis of the RNA used for the protein expression in *X. laevis* oocytes was performed using the *in vitro* RNA transcription kit mMessage mMachine SP6 (Ambion - Thermo Fisher Scientific). The plasmid that was used as a template for transcription was linearized and purified with phenol: Cl_3CH_3. The reaction was set as follows:

10 µl 2x NTP/CAP
1 µg template
x µl ddH_2O
2 µl buffer 10x
2 µl enzyme mix

20 µl total volume

The reaction was incubated for 60 min at 37°C, then 1 µl of DNase was added and the reaction was further incubated at 37°C for 15 min more. In order to precipitate the RNA, 30 µl of LiCl were added and the sample was kept over-night at -80°C. On the following day it was centrifuged at 4°C during 30 min at 17000 x g. The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged again under the same conditions. After the pellet was air-dried, it was resuspended in 20 µl of ddH_2O.

2.2.1.15. Pyrosequencing

Part of the sequence of PfCRT was amplified by PCR using one of the primers biotinylated (primers nº34 and nº35). The PCR product was mixed with 3 µl of Streptavidin

Sepharose HP (GE Healthcare) and 40 µl of binding buffer. The final volume was set to 80 µl. The mixture was transferred to a 96 well-plate V-bottom and shaken for 10 min at 1400 rpm. The strand separation was done using the PyroMark Q96 vacuum workstation as follows. The filter probes were flushed with 180 ml of ddH_2O to wash them and then they were lowered into the 96 well-plate containing the samples to capture the beads. The beads were washed with 70% ethanol by flushing the filter probes for 5 s, then denaturalized with the denaturation solution for 5 s and finally washed with wash buffer for 10 s. The filter probes were drain by raising the tool at 90° for 5 s and then the beads were released into a PyroMark Q96 Plate Low containing 0.4 µM of sequencing primer (primer nº36) in 40 µl of annealing buffer. The samples were heated at 80°C for 2 min and allowed to cool down to RT. The reagent cartridge was filled with the recommended volumes of PyroMark Gold Q96 Reagents provided by the software. The PyroMark Q96 Plate Low was placed on the heating block of the PyroMark Q96 ID and the cartridge on the dispensing unit. The set up and the run analysis were done using the PyroMark Q96 Software v1.0.

2.2.2. Methods in microbiology

2.2.2.1. Preparation of electrocompetent *E. coli* cells

One colony of PMC 103 cells was inoculated in 10 ml of SB and incubated over-night at 37°C with shaking at 250 rpm. The over-night culture (6 ml) was inoculated in 600 ml of SB and incubated for 3.5 h more. From this point on, the cells were always kept on ice. The culture was divided into 2 centrifuge bottles and centrifuged at 6000 x g during 20 min at 4°C. The supernatant was discarded and each pellet was resuspended in 300 ml of sterile ddH_2O and centrifuged again under the same conditions. The last step was repeated 2 times more. After the last centrifugation, the supernatant was discarded and each pellet was resuspended in 300 ml of sterile 10% (v/v) glycerol and centrifuged again. The supernatant was discarded and each of the pellets was resuspended in 1.2 ml of sterile 10% glycerol. The cells were aliquoted (50 µl aliquots) in 1.5 ml tubes on dry ice and immediately frozen at -80°C.

2.2.2.2. Transformation of competent *E. coli* cells

- PMC 103 electrocompetent cells

The cells (50 µl aliquots) were thawed on ice and mixed gently with 100 µl of 10% glycerol. The DNA of interest was mixed with the cells and the mix transferred to an

electroporation cuvette. The samples were electroporated at 2500 V and immediately 900 µl of prewarmed (37°C) SOC medium were added to the samples. The samples were transferred to 15 ml tubes and incubated at 37°C for 1h with shaking at 250 rpm. The cells were plated on LB agar plates containing the appropriate antibiotic concentration. The plates were incubated over-night at 37°C.

- XL10 Gold ultra-competent cells

The cells (40 µl aliquots) were thawed on ice and mixed gently. β-mercaptoethanol (1.2 µl) was added to each aliquot of cells. The cells were incubated on ice for 10 min and swirled every 2 min. The DNA of interest was mixed with the cells and the mix was incubated during 30 min on ice. The samples were subjected to a heat pulse at 42°C for 30 s. The tubes were incubated on ice for 2 minutes and then 900 µl of prewarmed (42°C) NZY+ broth were added to the samples. The samples were transferred to 15 ml tubes and incubated at 37°C for 1h with shaking at 250 rpm. The cells were plated on LB agar plates containing the appropriate antibiotic. The plates were incubated over-night at 37°C.

The antibiotic concentrations used were the following:

Ampicillin	100 µg/ml
Kanamicin	20 µg/ml
Chloramphenicol	34 µg/ml

2.2.2.3. Transformation of *S. cerevisiae*

One single colony was inoculated in 10 ml of YPDA and shaken over-night at 30°C. The culture was diluted to an OD_{600} of 0.4 in 25 ml of YPDA and grown for 3 h more. The cells were centrifuged at 1100 x g for 5 min and the pellet was resuspended in 20 ml of TE buffer. The cells were centrifuged again under the same conditions and the pellet was resuspended in 0.8 ml of 1X LiAc/0.5X TE buffer. The cells were incubated at RT for 10 min. For each transformation, 1 µg of plasmid and 100 µg of denatured salmon sperm DNA were mixed together with 100 µl of the yeast suspension. Then, 700 µl of 1X LiAc/40% PEG/1X TE buffer were added and mixed. The solution was incubated for 30 min at 30°C. DMSO (88 µl) was added to the mix, that was incubated at 42°C for 7 minutes. The cells were centrifuged for 10 s at 17000 x g and the supernatant was discarded. The cell pellet was resuspended in 1 ml of TE buffer and centrifuged again. Finally, the pellet was resuspended in 100 µl of TE buffer and plated on the appropriate selective plate.

2.2.2.4. Yeast Two Hybrid assay

- mRNA purification from total RNA

The mRNA purification from *P. falciparum* total RNA was performed using the FastTrack MAG mRNA isolation kit from Ambion according to the manufacturer's protocol. All the steps were performed using a magnetic particle separator. Briefly, FastTrack MAG Beads (100 µl) were washed twice and mixed with 150 µg of total RNA and 500 µl of binding buffer. The samples were incubated at 70°C for 5 min and then rotated for 10 min at RT. After three washing steps the beads were resuspended in 10 µl of ddH_2O and incubated at 37°C for 5 min to elute the mRNA. The mRNA was quantified and used directly for the synthesis of cDNA.

- cDNA synthesis

The cDNA synthesis from *P. falciparum* mRNA was performed using the Matchmaker Gold Yeast Two-Hybrid System kit from Clontech. The reaction was set up as follows:

2 µg mRNA
1 µl CDS primer
x µl ddH_2O

4 µl final volume

The mix was incubated at 72°C for 2 min and then placed on ice for 2 min. Next, the following reagents were added:

2 µl 5X First-Strand Buffer
1 µl dNTP Mix (10 mM)
1 µl DTT (100 mM)
1 µl SMART MMLV Reverse Transcriptase

9 µl final volume

The reaction was incubated for 10 min at 42°C. Then, 1 µl of SMART III-modified oligo was added and the reaction was mixed and incubated for 1h at 42°C. The sample was placed at 75°C for 10 min to end the first-strand synthesis. After that, it was cooled down to RT and 1 µl of RNAse H (2U) was added. The reaction was incubated for 20 min at 37°C and the cDNA was used as a template for a long distance PCR.

- Long distance PCR

Two 100 µl PCR reactions were set up for each experimental sample as follows:

2 µl First-Strand cDNA
70 µl ddH_2O
10 µl 10X Advantage 2 PCR Buffer
2 µl 50X dNTPs
2 µl 5' PCR primer
2 µl 3' PCR primer
10 µl 10X Melting Solution
2 µl 50X Advantage 2 Polymerase Mix

100 µl final volume

The program used in the thermocycler was the following:

95 °C 30 s
95 °C 10 s
68 °C 6 min* } 30 cycles
68 °C 5 min
4 °C ∞

* The thermocycler was programmed to increase the extension time by 5 s each successive cycle.

- cDNA purification

The cDNA after the long distance PCR was purified using a CHROMA SPIN TE-400 column for each of the 2 PCR samples. The columns were inverted in order to resuspend the gel matrix, the top cap and the break from the bottom of the column were removed and the columns were placed in a collection tube. They were centrifuged at 700 x g for 5 min and the equilibrium buffer was discarded. The PCR samples were applied to the center of the column and centrifuged under the same conditions. The purified samples were collected in a 1.5 ml tube. The 2 purified samples were combined and precipitated with sodium acetate / ethanol (1/10 volumes of 2.5M sodium acetate and 2.5 volumes of 100% ethanol). After ethanol precipitation the pellet was air-dried and resuspended in 20 µl of ddH_2O.

- Preparation of competent cells

One colony of a yeast strain expressing the appropriate prey was inoculated in 3 ml of YPDA and incubated 8-12 h at 30°C with shaking at 250 rpm. Next, 5 µl of the culture were inoculated in 250 ml of YPDA and incubated until the OD_{600} reached 0.3 (around 16-20 h). After this time, the cells were centrifuged at 700 x g during 5 min at RT. The supernatant was

discarded and the pellet was resuspended in 100 ml of fresh YPDA. The culture was incubated at 30°C until it reached an OD_{600} of 0.5 (around 5 h) and then divided into two 50 ml tubes and centrifuged at 700 x g for 5 min at RT. The supernatant was discarded and each pellet was resuspended in 30 ml of sterile ddH_2O. The cells were centrifuged again under the same conditions, the supernatant was discarded and each pellet was resuspended in 1.5 ml of 1.1xTE/LiAc. The cell suspension was transferred to a 1.5 ml tube and centrifuged at 17000 x g for 15 s. The supernatant was discarded and each pellet was resuspended in 600 µl of 1.1xTE/LiAc. The cells were used immediately for transformation.

- Library scale transformation

In a 15 ml tube, 5 µg of pGADT7 plasmid (SmaI-linearized) plus the 20 µl of the SMART-amplified cDNA and 200 µg of denatured yeastmaker carrier DNA were mixed together. Then 600 µl of fresh prepared competent cells and 2.5 ml of PEG/LiAc were added and the sample was mixed gently. The cells were incubated 45 min at 30°C. After that, 160 µl of DMSO were added to the mix and the cells were incubated at 42°C for 20 min. Next, the sample was centrifuged at 700 x g during 5 min, the supernatant was discarded and the pellet was resuspended in 3 ml of YPD Plus Medium. The cells were incubated at 30°C with shaking for 90 min and then centrifuged again under the same conditions. The supernatant was discarded and the cells were resuspended in 15 ml of 0.9% (w/v) NaCl Solution. The cells were plated in SD medium plates (-HIS, -LEU, -TRP; 1 ml/plate) and incubated for 3 days at 30°C.

2.2.3. Methods in protein biochemistry

2.2.3.1. Preparation of protein samples from *P. falciparum*

P. falciparum cultures with a parasitemia in trophozoites of 3-5% were purified using the MACS system. After elution of the iRBC, the samples were centrifuged (900 x g for 2 min) and the pellets were washed once with ice-cold PBS. The iRBCs were resuspended in the appropriate volume of protein lysis buffer for *P. falciparum* (approximately 1 ml for 50 µl of iRBC) and incubated on ice for 3 min. The samples were then centrifuged at 17000 x g for 1 min at 4°C and the pellet of parasites was washed 1-2 times with ice-cold PBS and kept at -80°C or resuspended directly in protein loading buffer.

2.2.3.2. SDS-PAGE electrophoresis

Protein samples were analyzed by SDS-PAGE electrophoresis (Shapiro et al., 1967). After the addition of the protein loading buffer, the protein samples were sonicated and heated at 70°C during 5 min before loading them into the gel. The PageRuler Plus Prestained (Ambion - Thermo Fisher Scientific) was used as a protein ladder. The gels were run at 40 mV (1 gel) or 80 mV (2 gels) during approximately 90 min in SDS-PAGE running buffer.

SDS-PAGE gels were prepared as follows:

Stacking gel	*Resolving gel*	
	10%	*12%*
3.46 ml ddH_2O	3.96 ml ddH_2O	3.35 ml ddH_2O
630 µl 1M Tris pH 6.8	2.5 ml 1M Tris pH 8.6	2.5 ml 1M Tris pH 8.6
50 µl 10% SDS	100 µl 10% SDS	100 µl 10% SDS
830 µl 30% acrylamide	3.33 ml 30% acrylamide	4 ml 30% acrylamide
50 µl 10% APS	100 µl 10% APS	100 µl 10% APS
5 µl TEMED	6 µl TEMED	6 µl TEMED

2.2.3.3. Coomassie staining of proteins

For protein visualization, the gels after SDS-PAGE electrophoresis were soaked in Coomassie solution for 10 min with constant shaking and then were incubated with Coomassie destaining solution until the protein bands could be clearly distinguished. The gels were kept in ddH_2O until they were dried using a gel drying system.

2.2.3.4. Western blotting

The SDS-PAGE gels were transferred to an Immun-Blot® PVDF membrane (Bio-Rad) using a Trans-blot SD semi-dry transfer cell. The membrane was activated soaking it in methanol for 30 s. The membrane and the SDS-PAGE gel were incubated in transfer buffer during 30 min prior to the transfer. Afterwards, 3 Whatman papers were soaked on transfer buffer and placed on the device, then the membrane was placed on top, then the SDS-PAGE gel and finally 3 more soaked Whatman papers. The transfer was run for 60 min at 230 mA. The membrane was blocked over-night with 5% (w/v) milk in PBS. The following day, the first antibody was diluted in 3% (w/v) BSA in PBS and the membrane was incubated with the solution during 60 min. Then, 3 washes of 10 min with PBST (0.1% (v/v) of Tween in PBS)

were performed. The membrane was blocked again with 5% (w/v) milk in PBS during 30 min. The incubation of the second antibody (diluted as well in 3% (w/v) BSA in PBS) was carried out during 30 min. Before the developing, the membrane was washed 3 times more during 10 min with PBST. The developing solution (BM chemiluminescence blotting substrate POD from Roche) was prepared fresh (2 ml of solution A + 20 µl of solution B) and the membrane was incubated with the solution for 5 min. Afterwards, the signal was captured with a blot scanner (C-DiGit from Li-Cor).

The dilutions of the antibodies that were used for Western blot are the following:

Anti-α-tubulin monoclonal	1:1000
Anti-BIP	1:2000
Anti-GFP monoclonal	1:1000
Anti-guinea pig-POD monoclonal	1:5000
Anti-HA tag monoclonal	1:1000
Anti-His tag monoclonal	1:1000
Anti-mouse-POD monoclonal	1:10000
Anti-PfCRT polyclonal	1:1000
Anti-rabbit-POD monoclonal	1:10000

2.2.3.5. Western blot stripping

For stripping of the antibodies, the Western blot membranes were incubated during 30 min with stripping buffer and then washed 3 times during 10 min with PBST (0.1% (v/v) of Tween in PBS).

2.2.3.6. Expression of recombinant GST-tagged PfCK2α and PfCK2α^{K72M}

Vectors for the *in vitro* expression of PfCK2α as well as for a mutated form of the protein (PfCK2α^{K72M}) were kindly provided by Prof. Christian Doerig. Both vectors are described in a publication by this group (Holland et al., 2009). Shortly, competent cells BL21 (DE3) were transformed with 0.5 µg of the pGEX-4T-3-PfCK2α or pGEX-4T-3-PfCK2α^{K72M}plasmids, plated in LB agar containing 100 µg/ml of ampicillin and incubated over-night at 37°C. A single colony was inoculated in 10 ml of LB and incubated at 37°C for 16 h with shaking at 200 rpm. The starter culture was diluted into 50 ml of SB medium to a starting OD_{600} = 0.1. The culture was incubated at 37°C and 200 rpm until the OD_{600} reached 0.5. The incubation temperature was reduced to 20°C for 20 min and then the

protein expression was induced by adding 0.1 mM IPTG. The expression continued for 20 h. The cells were harvested by centrifugation at 4000 x g at 4°C for 20 min. The pellet was stored at – 80°C until purification.

2.2.3.7. Purification of recombinant GST-tagged PfCK2α and PfCK2α^{K72M}

The protein pellet was resuspended in protein lysis buffer for *E. coli* (7.5 ml/g pellet) and homogenized by pipetting. The lysate was sonicated 3 times for 20 s on ice and was centrifuged at 17000 x g at 4°C during 30 min. In the meantime, 100 µl of glutathione beads were washed twice with 1 ml of ddH_2O and once with 1 ml of lysis buffer. The supernatant from the lysate and the glutathione beads were mixed in a 15 ml tube and rotated gently at 4°C during 2 h. Then, the beads were washed twice with 500 µl of lysis buffer and 3 times with wash buffer. All the centrifugation steps were carried out at 400 x g during 2 min. The bound protein was eluted in 200 µl of elution buffer. The protein samples were used on the kinase assays the same day of the purification when possible or aliquoted and frozen at -20°C after addition of 10% glycerol.

2.2.3.8. Expression of recombinant His-tagged PF11_0488$^{C\text{-terminal}}$

Competent BL21-CodonPlus-RIL cells were transformed with 0.5 µl of the pET28a-PF11_0488$^{C\text{-terminal}}$ plasmid, plated in LB agar containing 20 µg/ml of kanamicin and 34 µg/ml of chloramphenicol and incubated over-night at 37°C. A single colony was inoculated in 10 ml of LB and incubated at 37°C for 16 h with shaking at 200 rpm. The starter culture was diluted into 100 ml of SB medium to a starting OD_{600} = 0.1. The culture was incubated at 37°C and 200 rpm for one hour more and then the protein expression was induced by adding 0.05 mM IPTG. The temperature was reduced to 25°C and the culture was incubated for 4 h more at this temperature. The cells were harvested by centrifugation at 4000 x g at 4°C for 20 min. The pellet was stored at -80°C until purification.

2.2.3.9. Purification of recombinant His-tagged PF11_0488$^{C\text{-terminal}}$

The pellet was resuspended in wash buffer (5 ml/g pellet) and homogenized by pipetting. The lysate was sonicated 3 times for 20 s on ice and it was centrifuged at 17000 x g at 4°C during 30 min. In the meantime, 100 µl of cobalt charged resin (TALON™ from Clontech) were equilibrated. All the centrifugation steps were carried out at 400 x g during 2 min. The resin was centrifuged to remove the storage buffer and then washed twice

with 200 µl of wash buffer. The supernatant from the lysate and the cobalt resin were mixed in a 15 ml tube and rotated gently at 4°C during 30 min. Then the resin was washed 3 times with 200 µl of wash buffer. The bound protein was eluted in 100 µl of elution buffer. The protein samples were dialyzed 2 times for 90 min in 100 ml of dialysis buffer and used on the kinase assays the same day of the purification when possible or aliquoted and frozen at -20°C.

2.2.3.10. Immunoprecipitation of PfCRT-HA

Protein extracts were prepared from a *P. falciparum* strain (Dd2) transfected with the overexpression plasmid pARL-PfCRT^{Dd2}-HA. The protein pellet was resuspended in 3 volumes of RIPA buffer and incubated for 10 min at 4°C vortexing every 2 min. The samples were sonicated 2 times during 5 s and centrifuged at 17000 x g during 10 min at 4°C. The supernatant was diluted 10 times in NETT buffer and mixed with 25 µl (dried volume) of HA-agarose beads that were previously washed twice with NETT buffer. The sample was rotated gently in a wheel over-night at 4°C. The next day it was centrifuged (1600 x g for 3 min at 4°C) and the HA-agarose beads were washed as follows: 2 times during 3 minutes with 400 µl of NETT I buffer, 2 times during 3 minutes with 400 µl of NETT II buffer and 1 time during 3 minutes with 400 µl of low salt buffer. Alternatively, in order to dephosphorylate the protein, the last washing step was done with NEB buffer 4, the beads were resuspended in 50 µl of NEB buffer 4 and incubated at 37°C for 1h after the addition of 1 µl CIP. PfCRT^{Dd2}-HA was assayed on beads.

2.2.3.11. *In vitro* kinase assays

Standard kinase reactions were prepared in kinase assay buffer containing 3 µCi/sample of γ-ATP in a final volume of 50 µl. The reactions were carried out at 37°C for 30 min and were stopped by the addition of 50 µl of TCA 20%. The protein samples were incubated on ice for 30 min and centrifuged at 17000 x g for 10 min. The protein pellets were first washed with TCA 10% and then with pure acetone, air-dried and resuspended in 15 µl of protein loading buffer. The samples were separated by SDS-PAGE electrophoresis; the protein gels were dried using a gel drying system and exposed for autoradiography using XAR biomax films.

2.2.4. *Xenopus laevis* oocytes

2.2.4.1. *X. laevis* maintenance

Two year's old female *Xenopus laevis* frogs were purchased from NASCO and maintained by the animal facility of Heidelberg University (Interfakultär Biomedizinisches Forschungszentrum). The frogs were kept in aquariums at 18°C and were fed three times a week with food pellets.

2.2.4.2. Surgical isolation of ovaries from *X. laevis*

The frog was submerged in anesthetic solution during 15-30 min. When the frog could be turned upside down without showing any reaction, it was taken out of the solution and placed on a wet sheet of paper towel on the top of a metal surface on ice. The incision area was gently wiped with cotton soaked in 70% (v/v) ethanol. A small incision (1 cm) was made on the down left part of the abdomen. Both the skin and the muscular layer were cut. The ovary was pulled out from the incision and using tweezers and a scissor, small fragments of the ovary were cut and incubated in OR2 buffer. The incision in the muscular layer was closed using 1 or 2 stitches and the incision in the skin was closed afterwards using 2 or 3 stitches more. The frog was left to recover half covered in tap water, keeping the head over the water level. When fully awake, the frog was completely submersed in tap water and brought back to the aquarium. The frogs were operated a maximum of 5 times with a recovering period of minimum 3 months between each surgery.

2.2.4.3. Collagenase treatment

The ovary fragments were cut into small pieces, transferred to an Erlenmeyer flask and washed with OR2 until the solution was clear. The OR2 buffer was removed and the ovary fragments were incubated over-night at 16°C in collagenase solution with gentle agitation. The OR2 buffer does not contain Ca^{+2} in order to avoid the activation of proteases that results in oocyte damage (Goldin, 1992). The following morning the oocytes were washed five times with OR2 buffer and five times more with ND96 buffer. The oocytes were stored at 16°C in ND96 buffer.

2.2.4.4. Microinjection of *X. laevis* oocytes

The day following surgery, stage V and VI oocytes were manually selected for injection. Each oocyte was injected with 30 ng of RNA using a Nanoject II injector into the vegetal pole of the oocyte. The oocytes were then stored at 16°C in ND96 buffer. Every day the medium was exchanged and the damaged oocytes discarded.

2.2.4.5. Drug transport assays in *X. laevis* oocytes

Groups of 10 oocytes were incubated under each experimental condition. The oocytes were incubated during 60 min in uptake buffer plus the appropriate radioisotope and then washed 3 times in uptake buffer without radioisotope. Each individual oocyte was transferred to a scintillation vial. Then the oocytes were lysed adding 100 µl of a 10% (w/v) SDS solution and vortexing. Scintillation solution (2 ml) was added to each vial and the vials were shaken before the radioactivity of each sample was measured using a scintillation counter. Radioisotope concentrations as well as general conditions used on the assays can be found in Appendix III.

2.2.5. Methods in parasitology

2.2.5.1. *In vitro* culture of *P. falciparum*

Intraerythrocytic stages were maintained in continuous culture according to the general methodology used for *P. falciparum* (Trager and Jensen, 1976). The different strains were cultured *in vitro* at 3.5% hematocrit (group A^+) in complete RPMI/HEPES medium at 37°C under controlled atmospheric conditions: 5% O_2, 3% CO_2, 92% N_2 and 95% humidity. The parasites were fed at least every second day when the parasitemia was also determined using Giemsa-stained blood smears. The parasitemia of the cultures was maintained between 0.1% and 10% to ensure optimal growing conditions.

2.2.5.2. Freezing of *P. falciparum*

Parasite cultures, mainly ring stages, were resuspended and centrifuged at 900 x g for 2 min. The supernatant was discarded and, 1/3 of the pellet volume of freezing solution was added drop by drop and mixed carefully with the iRBCs. The samples were incubated at room temperature for 5 min and then 4/3 of freezing solution was added to the iRBCs drop

by drop. The samples were transferred to 2 cryogenic vials that were kept at -80°C for one night. For long term storage the samples were kept in liquid nitrogen.

2.2.5.3. Thawing of *P. falciparum*

The cryogenic vials were thawed in a 37°C water bath for 30 s. Drop by drop 0.2 ml of thawing solution I was added and the samples were transferred to a 15 ml tube. Again, very slowly, 9 ml of thawing solution II were added to the sample shaking time to time. The samples were centrifuged (900 x g for 2 min), the supernatant was removed, and slowly 7 ml of thawing solution III were added. The samples were centrifuged again under the same conditions, the supernatant was discarded and the pellets were resuspended in 14 ml of complete RPMI medium and transferred to a Petri dish containing 500 µl of RBC.

2.2.5.4. Synchronization of *P. falciparum*

The parasites were synchronized in ring stages using sorbitol 5% as described previously (Lambros and Vanderberg, 1979). This method is based on the differential permeability of RBC infected by mature forms of the parasite which are permeable to sorbitol and are killed by osmotic shock. The cultures were resuspended and centrifuged at 900 x g for 2 min and the supernatant was discarded. The pellet was resuspended in 8 ml of prewarmed sorbitol 5% and incubated at 37°C for 5 min. The cells were centrifuged at 900 x g for 2 min, the supernatant was discarded and the pellet was resuspended in 14 ml of complete RPMI medium and transferred back to a Petri dish.

2.2.5.5. Magnetic purification

P. falciparum trophozoite and schizont stages were purified using the MACS system that takes advantage of the paramagnetic properties of hemozoin to facilitate the purification of late stage parasites by magnetic cell sorting (Paul et al, 1981). The MACS CS column was washed twice with MACS buffer and inserted into the VarioMACS separator. The cultures were resuspended and applied to the top of the column. The flow was adjusted to 1 drop every 3 s. The column was washed with MACS buffer until the flow-through was clear. The column was removed from the separator and the enriched late stages iRBC were eluted in 10 ml of MACS buffer. The cells were centrifuged at 700 x g for 2 min and the pellet was resuspended in the appropriate buffer according to the experiment the cells were going to be used for.

2.2.5.6. Transfection of *P. falciparum*

P. falciparum ring stages were transfected by electroporation as described previously (Wu et al., 1995). The plasmids that were going to be transfected (75 µg) were precipitated with sodium acetate / ethanol (1/10 volumes of 2.5 M sodium acetate and 2.5 volumes of 100% ethanol). After the ethanol precipitation and one washing step with ethanol 70%, the pellet was air-dried and resuspended in 30 µl of TE buffer. When transfecting with PCR products, 8 x 50 µl PCR reactions were combined and precipitated with ammonium acetate / ethanol (0.5 volumes of 2.5M ammonium acetate and 2.5 volumes of 100% ethanol). After ethanol precipitation and one wash with ethanol 70%, the pellet was air-dried and resuspended in TE buffer. The DNA concentration was quantified and 30 µg were used for transfection. Cytomix (370 µl) was added to each of the DNA samples.

Synchronized *P. falciparum* cultures with a parasitemia of 3-5% ring-stage parasites were centrifuged at 900 x g during 2 min. The supernatant was discarded and 200 µl of pellet were transferred to a new tube. The cytomix/plasmid mix was added to the pellet of iRBC and mixed gently. The sample was transferred to an electroporation cuvette and electroporated at 310 V and 950 µF. Immediately, the electroporated sample was transferred to a Petri dish containing 14 ml of transfection medium and 500 µl of RBCs.

After the day of transfection, the medium was changed every day during 7 days. The second day after the transfection, the appropriate drug for selection was added to the medium. After the first week, the medium was changed every 2 days. Fresh RBCs (100 µl) were added once a week until the transfectant parasites appeared.

The concentrations of the drugs used are the following:

	Stock	*Final*
DSM1	10 mM	1.5 µM
WR99210	20 µM	5 nM
Blasticidin	10 mg/ml	3.8 µg/ml
Shield-1	0.5 mM	200 nM

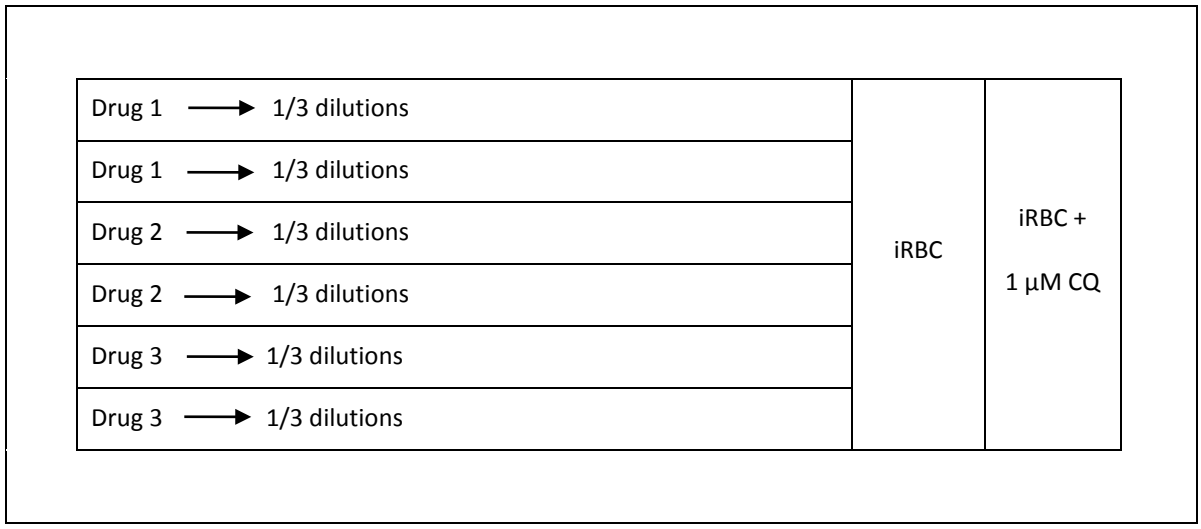
2.2.5.7. IC_{50} determination

The IC_{50} determinations were performed according to the standard SYBR green fluorescence-based assay (Smilkstein et al., 2004). It is the gold standard protocol used to measure the effectiveness of a drug to inhibit parasite growth. The parasites that were going

to be used on the assay were synchronized two days before the experiment. The day of the experiment the culture was adjusted to 0.5% parasitemia and 3% hematocrit.

From a 96 well plate, the first and the last rows and columns were not used on the assay; they were just filled with 100 µl of RPMI medium to avoid evaporation. The tenth column was used as a positive control (100% growth, no drug) and the eleventh column as a negative control (0% growth, 1 µM CQ). Rows 2-7 were used to do duplicates of the assay for each drug. The highest drug concentration to be used on the assay (75 µl) was pipetted on the second column and 1/3 serial dilutions were performed (25 µl of the first well to the next well containing 50 µl of RPMI complete medium and so on).

Plate scheme:



The drug concentrations used on the assays were the following (final concentration on the first well):

CQ: 1.5 µM
QN: 4.5 µM
ML-7: 50 µM

The parasites (50 µl) were added to each well and the plates were incubated during 72 hours at 37°C. After the incubation time, the plates were frozen at -80°C at least for 2 h. The plates were thawed for at least 1h and 100 µl of lysis buffer containing SYBR green (1.2 µl in 10 ml of buffer) were added to each well. The plates were shaken briefly and incubated for at least 1 h at RT. Fluorescence measurements were done using the plate reader FLUOstar OPTIMA and the following parameters: excitation wavelength: 485 nm; emission wavelength: 520 nm; gain: 1380; nº of flashes/well: 10; top optic.

2.2.5.8. Drug transport assays in *P. falciparum*

Drug transport assays were performed according to the methodology established in the lab (Sanchez et al., 2003). *P. falciparum* cultures with a parasitemia in young trophozoites of ~3% were purified with the MACS system. The cultures were synchronized with sorbitol 5% the previous day and fed at least 20 min before starting the purification. After purification, the cells were centrifuged and resuspended in RMPI medium without bicarbonate equilibrated at 37°C at pH 7.3. The concentration of the parasites was adjusted between 20000 and 30000 iRBC/µl. The appropriate amount of radiolabelled drug (40 nM CQ or 20 nM QN) was added to the cells that were incubated at 37°C during specific time periods. Every 5 min, 2 µl of 0.5 M glucose were added to the cells. From each sample, two 75 µl aliquots were transferred to a PCR tube containing 100 µl of separation oil (a 5:4 mixture of dibutylphthalate and diocytlphthalate) and 75 µl of RMPI medium without bicarbonate equilibrated at 4°C at pH 7.3. The PCR tubes were placed inside 1.5 ml tubes without lid and kept on ice. The samples were immediately centrifuged at 17000 x g for 1 min and then 75 µl of the upper phase of each duplicate were transferred to a scintillation vial containing 2 ml of scintillation cocktail. The tip of each PCR tube was cut with a scalpel and transferred to a new 1.5 ml tube. Next, 100 µl of tissue solubilizer (2:1 mixture of ethanol and tissue solubilizer from Pharmacia) were added to the tube and the samples were incubated over-night at 55°C. The following day, 25 µl of H_2O_2 (30%) were added to the solubilized pellets until the color was bleached. HCl (25 µl) was then added in order to neutralize the samples. The lids of the 1.5 ml tubes were cut and each tube was transferred to a scintillation vial containing 4 ml of scintillation cocktail. The radioactivity of the upper phase and the pellets was measured using a scintillation counter.

2.2.6. Microscopy methods

2.2.6.1. IFA of *X. laevis* oocytes

All the incubation steps were performed in gentle agitation. After 2 days of expression, the oocytes were fixed during 4 h using paraformaldehyde 4% (v/v) and then washed 3 times during 5 min with 3% (w/v) BSA in PBS. Afterwards the oocytes were permeabilized during 60 min in 0.05% (w/v) NP40 in PBS. Next, another 3 washing steps were performed as before. The incubation with the first antibody was performed over-night at 4°C. The antibodies were diluted in 3% (w/v) BSA in PBS. On the following day, 3 more

washing steps were performed as before. Finally, the oocytes were incubated during 3 h with the secondary antibody and washed again 3 times. The oocytes were analyzed using the confocal microscope LSM 510.

The dilutions of the antibodies that were used for the IFAs are the following:

Anti-His tag monoclonal	1:1000
Anti-InsP_3R-II polyclonal	1:50
Anti-mouse Alexa Fluor 488	1:1000
Anti-rabbit Alexa Fluor 546	1:1000

2.2.6.2. IFA of *P. falciparum*

The parasites were purified using the MACS system and washed once with PBS. The samples were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min. The samples were centrifuged (all the centrifugations were carried out at 600 x g for 2 min) and the pellets were resuspended and incubated with 0.1% (v/v) Triton in PBS during 15 min. After a washing step with PBS, the samples were neutralized during 10 min with 10 mM NH_4Cl pH 7.0. Then the iRBCs were washed 1 time with PBS and one time with 3% (w/v) BSA in PBS. The 3% (w/v) BSA in PBS solution was used for the blocking and the rest of washing steps as well as to dilute the antibodies. The samples were blocked for 2 h and afterwards the incubation with the first antibody was carried out for 90 min. Then the iRBCs were washed 3 times and incubated with the secondary antibody for 45 min. The samples were then washed again 3 times and kept over-night in PBS at 4°C until they were analyzed using the confocal microscope LSM 510.

The dilutions of the antibodies that were used for the IFAs are the following:

Anti-HA tag monoclonal	1:1000
Anti-PfCRT polyclonal	1:1000
Anti-mouse Alexa Fluor 488	1:1000

2.2.6.2. Confocal fluorescence microscopy

Parasites and oocytes labeled by IFA and live GFP tagged parasites were analyzed using the LSM 510 laser scanning microscope. For live imaging, the parasites were purified using the MACS system, washed twice in ringer solution and imaged in a perfusion chamber. The samples labeled with anti-mouse Alexa Fluor 488 and the GFP-tagged proteins were

excited using a 488 nm Argon ion laser and the emission was detected using a 505-550 nm band pass filter. The samples labeled with anti-rabbit Alexa Fluor 546 were excited using a 543 nm Helium-Neon laser and the emission was detected using a 560 nm low pass filter. The objective used was a C-Apochromat with 100 x magnification. The images were acquired using the LSM imaging software and processed with the FIJI program.

2.2.8. Data analysis

All the data analysis was performed using SigmaPlot 11.0.

3. Results

3.1. PFE0825w characterization in *X. laevis* oocytes

3.1.1. *Pfe0825w* has alternative mRNA splice variants

The amplification of *pfe0825w* from cDNA revealed that this gene is transcribed into three different mRNA variants. Variant 0 was identified in Henry Vial's group (CNRS, Montpellier) from 3D7 cDNA. It follows the genomic organization predicted by PlasmoDB with 6 exons and 5 introns. Two other mRNA variants were identified from 3D7, 7G8 and GB4 cDNA. The fourth exon is not present in variant 1, while in variant 2 the third intron is not spliced out (fig. 3.1). As a result, variant 1 and 2 code for ORFs with alternative start codons downstream of the ATG starting codon in variant 0 (see appendix II). No differences in the amino acid sequences between 7G8 and GB4 were detected.

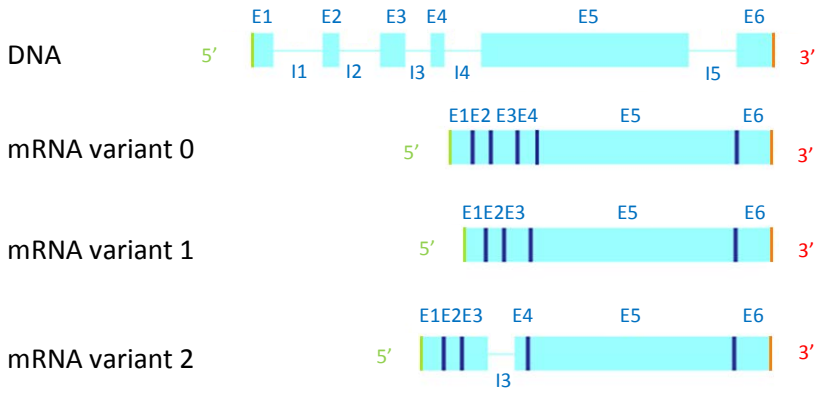


Figure 3.1. Alternative mRNA splicing variants of pfe0825w.

Diagram of the pfe0825w predicted coding sequence in PlasmoDB (DNA) and experimentally identified mRNA sequences (mRNA variants). Exons are represented by light blue boxes (E1-E6) and introns by light blue lines (I1-I5).

3.1.2. PFE0825w localizes at the oolemma of PFE0825w-his-expressing *X. laevis* oocytes

The codon-optimized sequences of the three mRNA variants whose putative endosomal-lysosomal trafficking motifs were substituted by alanines (see appendix II) were cloned into the expression vector pSP64T (see appendix I) and transcribed *in vitro*. Expression of PfCRT in *X. laevis* oocytes was reported to be improved by removing such trafficking motifs from both termini of the PfCRT protein sequence (Martin et al., 2009). The

coding region of variant 2 that is not in frame in the other variants was cloned from *P. falciparum* gDNA and was not codon optimized (see yellow sequence in appendix II). All sequences were fused to a 6xHis tag in order to study the localization of the transporter when expressed in *X. laevis* oocytes. PFE0825w expression was examined by confocal fluorescence imaging of fixed *pfe0825w-his*-injected oocytes and water-injected oocytes. Variants 0 and 1 showed a co-localized expression with the inositol 1,4,5 triphosphate receptor ($InsP_3R$), a receptor localized at the oocyte oolemma (Parys et al., 1992). The expression of variant 2 was not detected (fig. 3.2).

A

N-terminal

PFE0825w *P. falciparum*	1 MEVTSTLLEK GKNFAQDPSE VFPESKKFFF 30
PFE0825w motif free	1 MEVTSTLLEK GKNFAQDPSE AAPESKKAAF 30

C-terminal

PFE0825w *P. falciparum*	422 IFKDDKDSIE TMFSSIKSIL 442
PFE0825w motif free	422 IFKAAKASIA TMFSSIKSIL 442

B

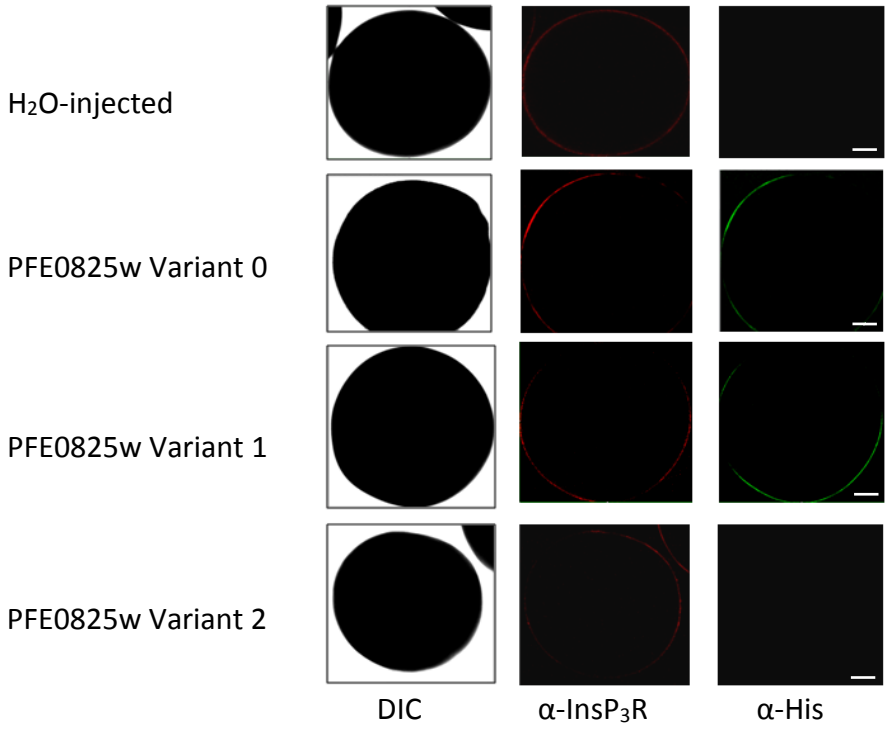


Figure 3.2. PFE0825w expression at the oolemma of X. laevis oocytes.

A. *Mutagenesis of the putative trafficking motifs of PFE0825w. The putative trafficking motifs of the N-terminal and C-terminal domains of PFE0825w were substituted by alanines (highlighted in green) to promote the correct trafficking of the protein to the oocyte oolemma.* ***B.*** *Immunofluorescence of fixed H_2O-injected and pfe0825w-His-injected oocytes using a mouse monoclonal anti-His antibody and rabbit polyclonal anti-$InsP_3R$ antibodies. The oocytes were injected with 30 ng of RNA or water and incubated for 2 days at 18°C in ND96 buffer. Scale bar: 200 µm.*

3.1.3. PFE0825w does not transport [^{14}C]-TEA, [^{3}H]-MPP, [^{3}H]-choline or [^{14}C]-T3 under the experimental conditions used

PFE0825w is predicted to be an organic cation transporter and therefore, the first approach in this study was to use [^{14}C]-TEA as a substrate in order to characterize its transport properties. This compound has been widely used in other studies to characterize the transport properties of several organic cation transporters (Roth et al., 2012). In accordance with the expression data, only *X. laevis* oocytes injected with mRNA from *pfe0825w* variant 0 (V0) and variant 1 (V1) were further analyzed regarding their transport properties. Preliminary uptake experiments were conducted at a wide pH range (5 to 7.3) as well as substrate concentrations (20 to 320 µM) in order to analyze the best experimental settings. No significant difference in uptake was observed between the water-injected oocytes and the PFE0825W-his-expressing oocytes under any of the tested conditions (fig. 3.3). Different RNA amounts (10-50 ng) were also injected but not further positive outcome was obtained (data not shown).

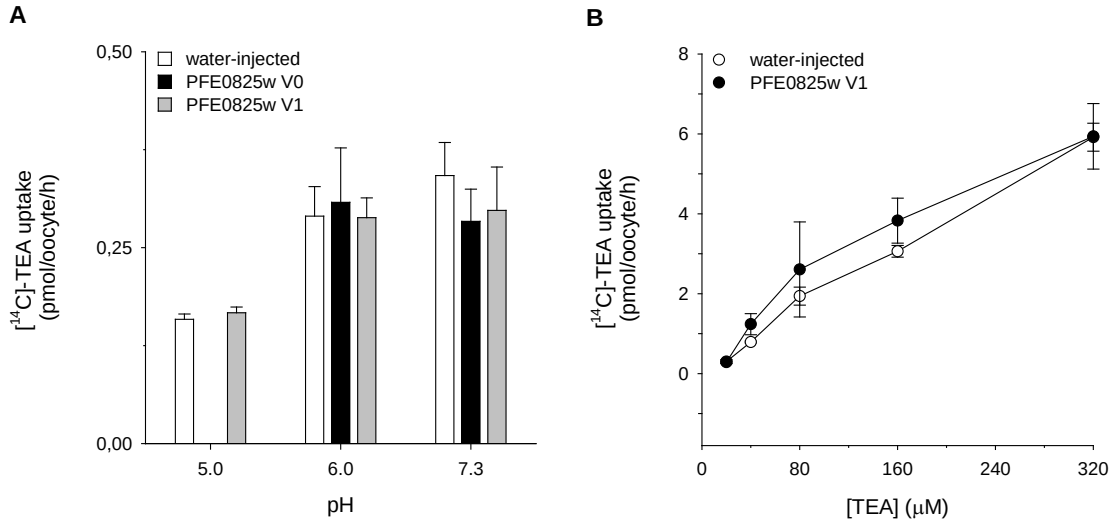


Figure 3.3. TEA accumulation by PFE0825-his-expressing X. laevis oocytes.

A. *Effect of pH on [^{14}C]-TEA accumulation by PFE0825w-his-expressing X. laevis oocytes. Two days post-injection oocytes were incubated for 60 min at 19°C in uptake buffer containing 20 µM [^{14}C]-TEA, adjusted to different pHs. The data represent the mean ± SEM of one to five independent determinations with 10 oocytes per condition* ***B.*** *Effect of TEA concentration on [^{14}C]-TEA accumulation by PFE0825w-his-expressing X. laevis oocytes. Two days post-injection oocytes were incubated for 60 min at 19°C in uptake buffer (pH 6.0) containing 20 µM [^{14}C]-TEA + different concentrations of cold TEA (0, 20, 60, 140 and 300 µM). The data represent the mean ± SEM of four to eleven independent determinations with 10 oocytes per condition. No significant difference was observed between water-injected and PFE0825w-his-expressing oocytes for any of the conditions.*

To exclude the possibility of TEA being a poor substrate of PFE0825w, the compounds [^{3}H]-MPP, [^{3}H]-CQ and [^{3}H]-choline were tested under the same experimental settings.

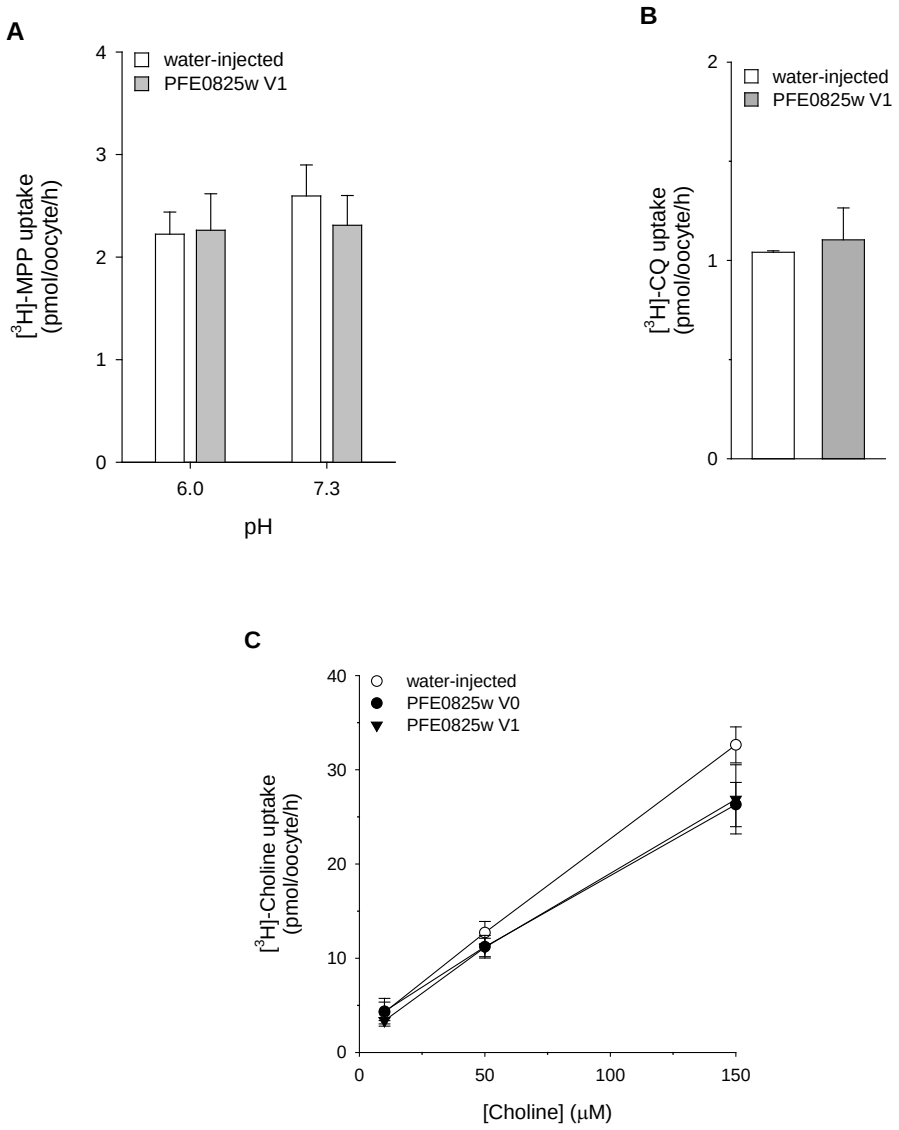


Figure 3.4. MPP, CQ and Choline accumulation by PFE0825w-his-expressing X. laevis oocytes.

A. *Effect of pH on [^{3}H]-MPP accumulation by PFE0825w-his-expressing X. laevis oocytes. Two days post-injection oocytes were incubated for 60 min at 19°C in uptake buffer containing 100 µM MPP adjusted to different pHs. The data represent the mean ± SEM of four independent determinations with 10 oocytes per condition.* ***B.*** *[^{3}H]-CQ accumulation by PFE0825w-his-expressing X. laevis oocytes. Two days post-injection oocytes were incubated for 60 min at 19°C in uptake buffer containing 10 µM CQ. The data represent the mean ± SEM of two independent determinations with 10 oocytes per condition.* ***C.*** *Effect of choline concentration on [^{3}H]-choline accumulation by PFE0825w-his-expressing X. laevis oocytes. Two days post-injection oocytes were incubated for 60 min at 19°C in uptake buffer (pH 6.0) containing different concentrations of choline (10, 50 and 150 µM). The data represent the mean ± SEM of three independent determinations with 10 oocytes per condition. No significant difference was observed between water-injected and PFE0825w-his-expressing oocytes for any of the conditions.*

In similar approaches, MPP and choline have shown to be appropriate substrates of the organic cation transporters hCHT1, rOCT1 and hOCT1 (Busch et al., 1996; Okuda and Haga, 2000; Zhang et al., 1997). As shown in Figure 3.4, MPP, CQ and choline were also not differentially taken up by PFE0825w-his-expressing oocytes under any of the experimental conditions tested.

Due to the lack of activity observed in the previous experiments and to rule out a detrimental effect of the His tag on the activity of the transporter, as previously reported for other proteins (Perron-Savard et al., 2005; Sabaty et al., 2013), the tag was removed from the constructs and further uptake experiments were performed.

In addition, some modifications were applied to the uptake protocol in order to overcome the lack of transport activity. The concentration of radiolabelled [^{14}C]-TEA was increased up to 50 µM and the final concentration of TEA was set to 200 µM. The assay temperature was increased to 30°C, as it was previously reported for PfCRT that an increase in temperature during the uptake experiments improved the activity of the transporter (Summers and Martin, 2010). As a final approach, the incubation temperature of the oocytes during the two days of expression time was reduced to 14°C in order to facilitate the correct folding of the transporter and therefore its functional expression.

The uptake of the transporter's putative substrate [^{14}C]-T3 was also tested under these conditions. Additionally, the PfCRT-mediated [^{3}H]-CQ uptake was conducted in parallel as a positive control for the experiments.

Despite the modifications on the uptake protocol, no differences were detected between water-injected oocytes and PFE0825w-expressing oocytes for any of the compounds used on the experiments. On the other hand, *pfcrt*-injected oocytes accumulated 4 times more chloroquine than water-injected oocytes, proving that the quality of the oocytes and the experimental procedures used were adequate.

Appendix III contains a detailed list of all the different experimental conditions tested for each PFE0825w variant.

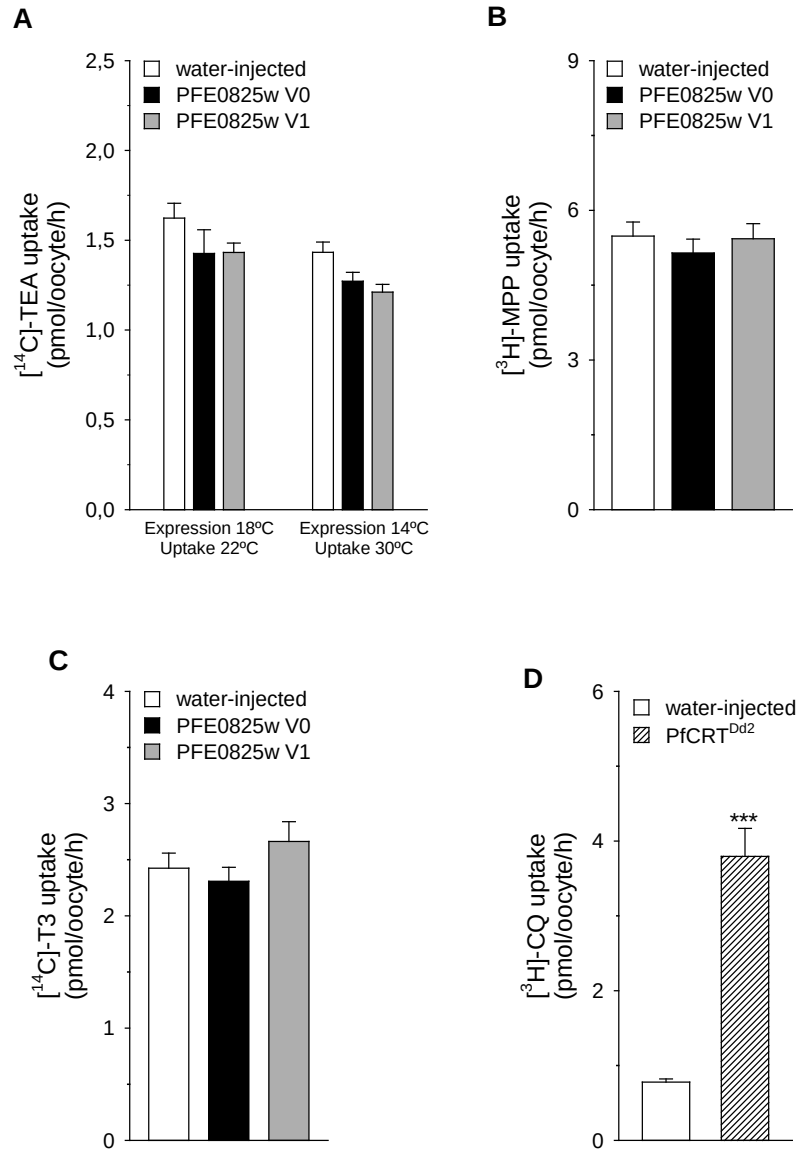


Figure 3.5. TEA, MPP and T3 accumulation by PFE0825w-expressing X. laevis oocytes and CQ accumulation by PfCRT-expressing X. laevis oocytes.

A. *Effect of expression and uptake temperatures on [^{14}C]-TEA accumulation by PFE0825w-expressing X. laevis oocytes. After RNA injection, the oocytes were incubated at 18°C or 14°C and after two days of expression, the oocytes were incubated for 60 min at 22°C or 30°C in uptake buffer (pH 6.0) containing 200 µM TEA.* ***B.*** *[^{3}H]-MPP accumulation by PFE0825w-expressing X. laevis oocytes. Three days post-injection oocytes were incubated for 60 min at 30°C in uptake buffer (pH 6.0) containing 100 µM MPP.* ***C.*** *[^{14}C]-T3 accumulation by PFE0825w-expressing X. laevis oocytes. Three days post-injection oocytes were incubated for 60 min at 30°C in uptake buffer (pH 6.0) containing 200 µM T3.* ***D.*** *[^{3}H]-CQ accumulation by PfCRT-expressing X. laevis oocytes. Three days post-injection oocytes were incubated for 60 min at 22°C in uptake buffer (pH 6.0) containing 10 µM CQ. All the data represent the mean ± SEM of one determination with 10 oocytes per condition. No significant difference was observed between water-injected and PFE0825w-his-expressing oocytes for any of the substrates. The difference in CQ uptake between water-injected and PfCRT-expressing oocytes was assessed using the Mann-Whitney Rank Sum test; p<0.001 (***).*

3.2. Analysis of the role of phosphorylation in the drug-resistance-mediating function of the chloroquine resistance transporter PfCRT

Different experimental approaches were followed in order to achieve the aims of the project. On the one hand, the identification of the kinase that phosphorylates PfCRT was attemped through two different strategies: a screen to identify kinase inhibitors with an effect on CQ accumulation and a two yeast hybrid assay. On the other hand, PfCRT phosphorylated residues were mutated to alanine using the CRISPR-Cas9 system in order to study the direct role of phosphorylation in PfCRT-mediated drug transport.

3.2.1. The kinase inhibitor ML-7 modulates CQ accumulation and CQ and QN susceptibility

The 411 and 416 phosphorylation sites of PfCRT were identified as consensus phosphorylation recognition sequences of CK2 using the PhosphoMotif Finder online tool (Amanchy et al., 2007). No motif was identified for the phosphorylated sequence at position 33.

Position	Sequence	Motif	Motif features
33	SRLG	-	-
411	SegE	[S/T]-XX-[D/E]	Casein kinase II substrate motif
416	TnvD	[S/T]-XX-[D/E]	Casein kinase II substrate motif

Table 3.1. PfCRT phosphorylation motifs.

Motifs identified using the PhophoMotif Finder online tool.

Taking this prediction into account, a screen of more than 25 different compounds known to affect phosphorylation events by targeting different classes of kinases and phosphatases was performed in the lab by Dr. Cecilia Sanchez. Three known *P. falciparum* casein kinase II (CK2) inhibitors ML-7, rottlerin and TBB, as well as several human CK2 inhibitors (Perez et al., 2011) were included in the screen.

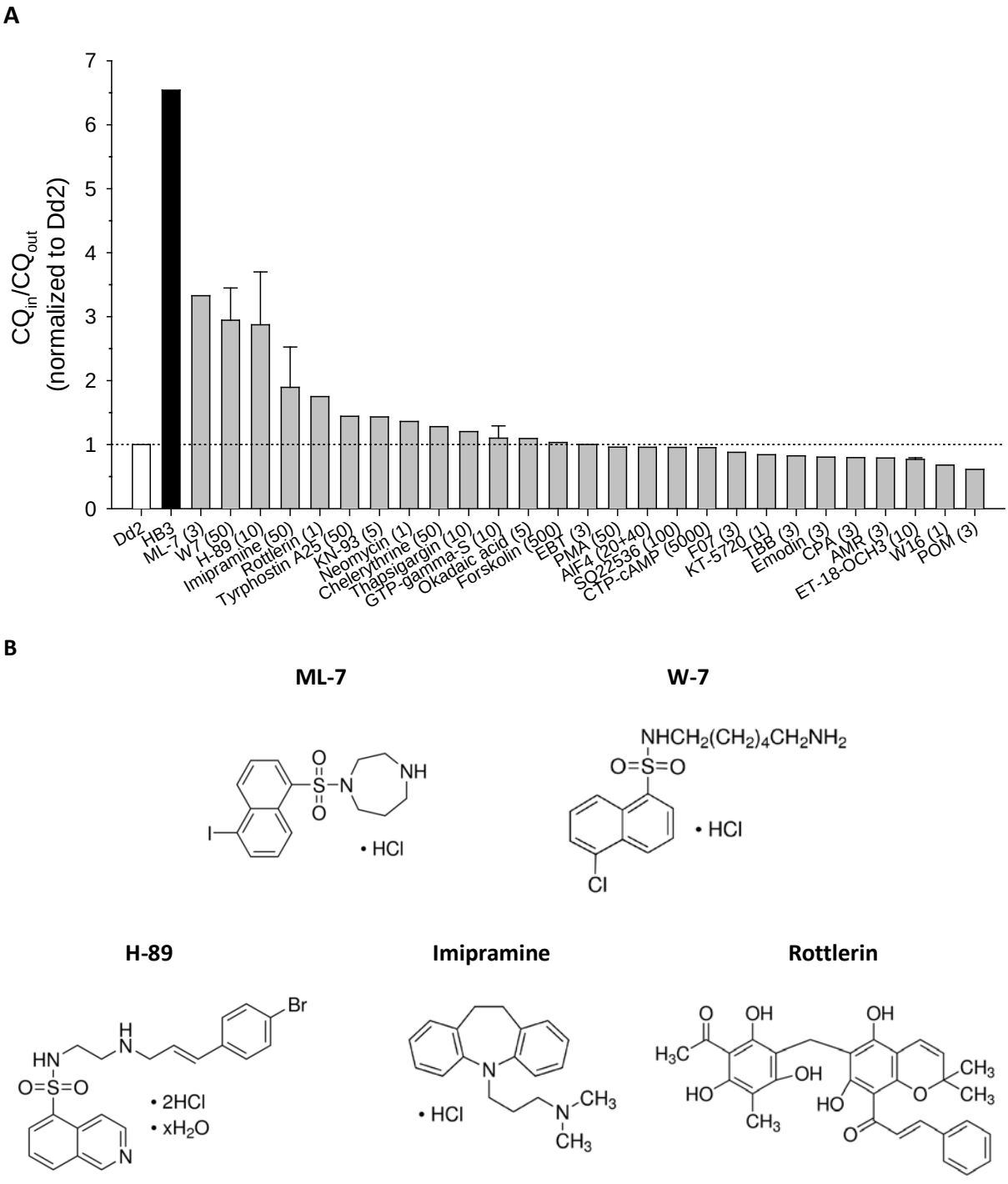


Figure 3.6. Effect of kinase and phosphatases inhibitors on CQ accumulation in P. falciparum.

A. *Chloroquine accumulation in Dd2 after 5 min of treatment with different compounds known to affect phosphorylation. The maximum concentration of each compound used in the assay is indicated in brackets (µM). The accumulation data are normalized to the CQ accumulation in untreated Dd2. The data represent one single determination or at least two determinations when error bars (SEM) are shown. These results were generated by Dr. Cecilia Sanchez.* ***B.*** *Chemical structure of the 5 compounds with higher effect on CQ accumulation.*

The readout of the assay was CQ accumulation. To compare the level of drug accumulation between the treated and the untreated Dd2 strain, the parasites were incubated with radiolabelled CQ and the ratio between the drug concentration within the infected erythrocytes and the extracellular medium was calculated at the 5 min time point. The same approach has been used in numerous studies in order to correlate CQ and QN susceptibility with CQ and QN efflux outside the food vacuole (Sanchez et al., 2003; Lakshmanan et al., 2005) as well as to characterize the transport mechanisms of different drugs in *P. falciparum* (Wein et al., 2014).

In Figure 3.6, CQ accumulation of Dd2 parasites treated with the different compounds at the maximum concentration used in the assay is shown normalized to CQ accumulation of untreated Dd2. The screen revealed that the Dd2 strain treated with the compounds rottlerin, ML-7, W7, H-89 and imipramine accumulated higher levels of CQ compared with the untreated Dd2. As mentioned before, rottlerin and ML-7 are known inhibitors of PfCK2 (Holland et al., 2009). The role of these two inhibitors in relation to CQ susceptibility was further investigated in this study. CQ and QN accumulation was measured in the sensitive strain HB3 and the resistant strain Dd2 after 5 minutes of treatment with ML-7 and rottlerin in a dose-response curve.

ML-7 showed the same profile as verapamil, a widely known CQ chemosensitizer, in the same experimental procedure (Sanchez et al., 2004). At increasing concentrations of the inhibitor, the CQR strain Dd2 accumulated more CQ than the untreated strain, whereas there was no effect on the CQS strain HB3 at the same concentrations. The inhibitor showed its highest activity in Dd2 at 10 µM although, at this concentration, the CQ accumulation values for HB3 decreased, indicating a toxic effect at high concentrations of the inhibitor. On the other hand, rottlerin showed a lower effect at the same concentration range for Dd2 and a higher toxic effect on HB3. Regarding QN accumulation, the effect of both kinase inhibitors was the same for both CQR and CQS strains, suggesting no specific activity. Remarkably, rottlerin and ML-7 are inhibitors of PfCK2 in the same µM range: ML-7 inhibits the enzyme with an IC_{50} of roughly 3 to 4 µM and rottlerin exhibits an IC_{50} of 7 µM (Holland et al., 2009). Besides, ML-7 was previously used as an inhibitor of the myosin light chain kinase in other studies with the concentrations ranging from 1 to 40 µM, comparable to the conditions used in this study (Arii et al., 2010; Connell and Helfman, 2006; Lin et al., 2012).

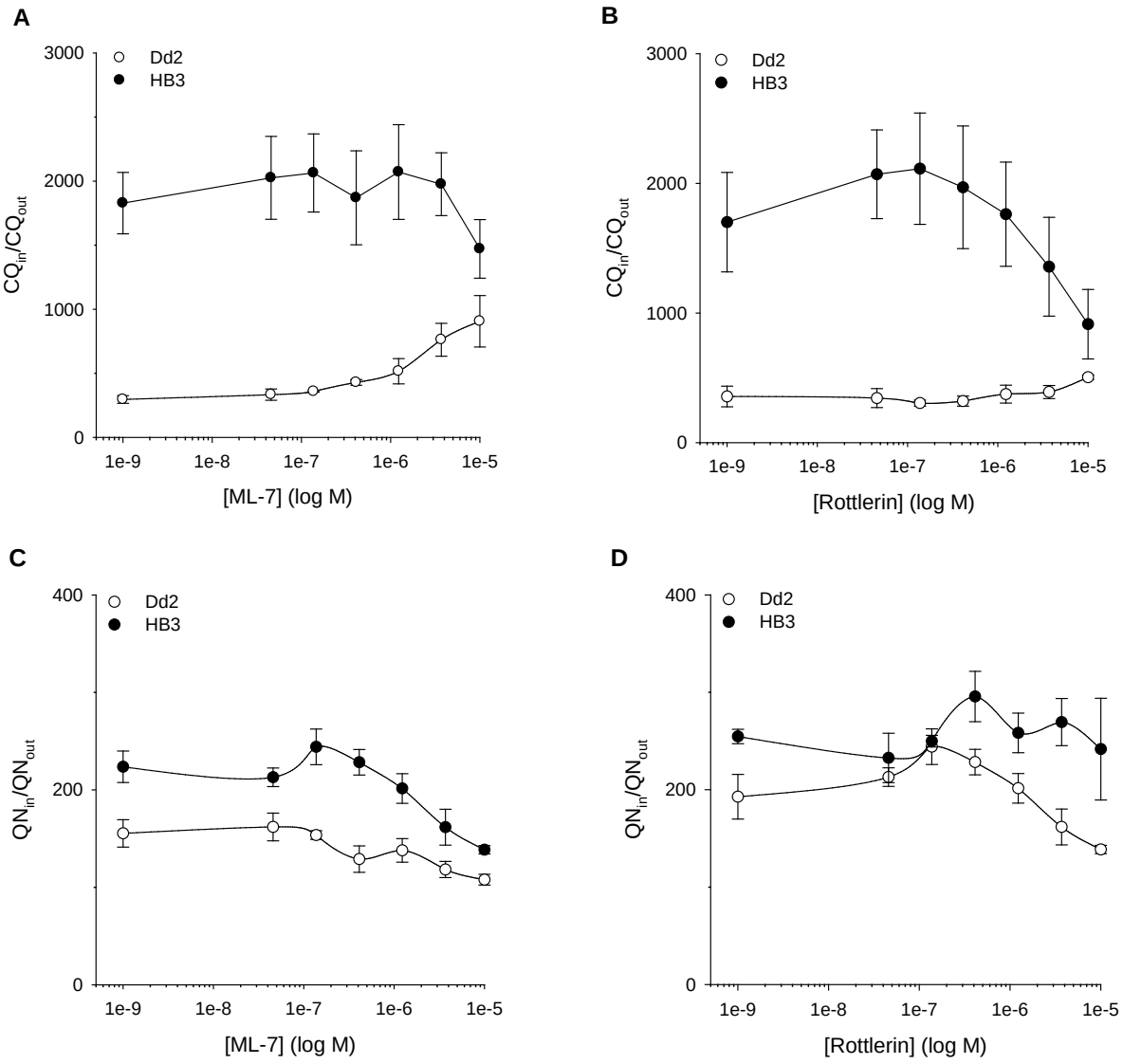


Figure 3.7. Effect of rottlerin and ML-7 on CQ and QN accumulation in P. falciparum.

A. *Chloroquine accumulation in Dd2 and HB3 treated with different concentrations of ML-7 at the 5 minute time point.* ***B.*** *Chloroquine accumulation in Dd2 and HB3 treated with different concentrations of rottlerin at the 5 minute time point.* ***C.*** *Quinine accumulation in Dd2 and HB3 treated with different concentrations of ML-7 at the 5 minute time point.* ***D.*** *Quinine accumulation in Dd2 and HB3 treated with different concentrations of rottlerin at the 5 minute time point. The data represent the mean ± SEM of three to four independent determinations.*

The intrinsic antimalarial activity of ML-7 was also determined by calculating the IC_{50} value of this compound in the Dd2 and HB3 strains. ML-7 only had an effect on parasite growth at high concentrations. In both cases, the ML-7 IC_{50} value was higher than 50 µM, although the Dd2 strain is shown to be more sensitive to this compound than HB3 (figure 3.8).

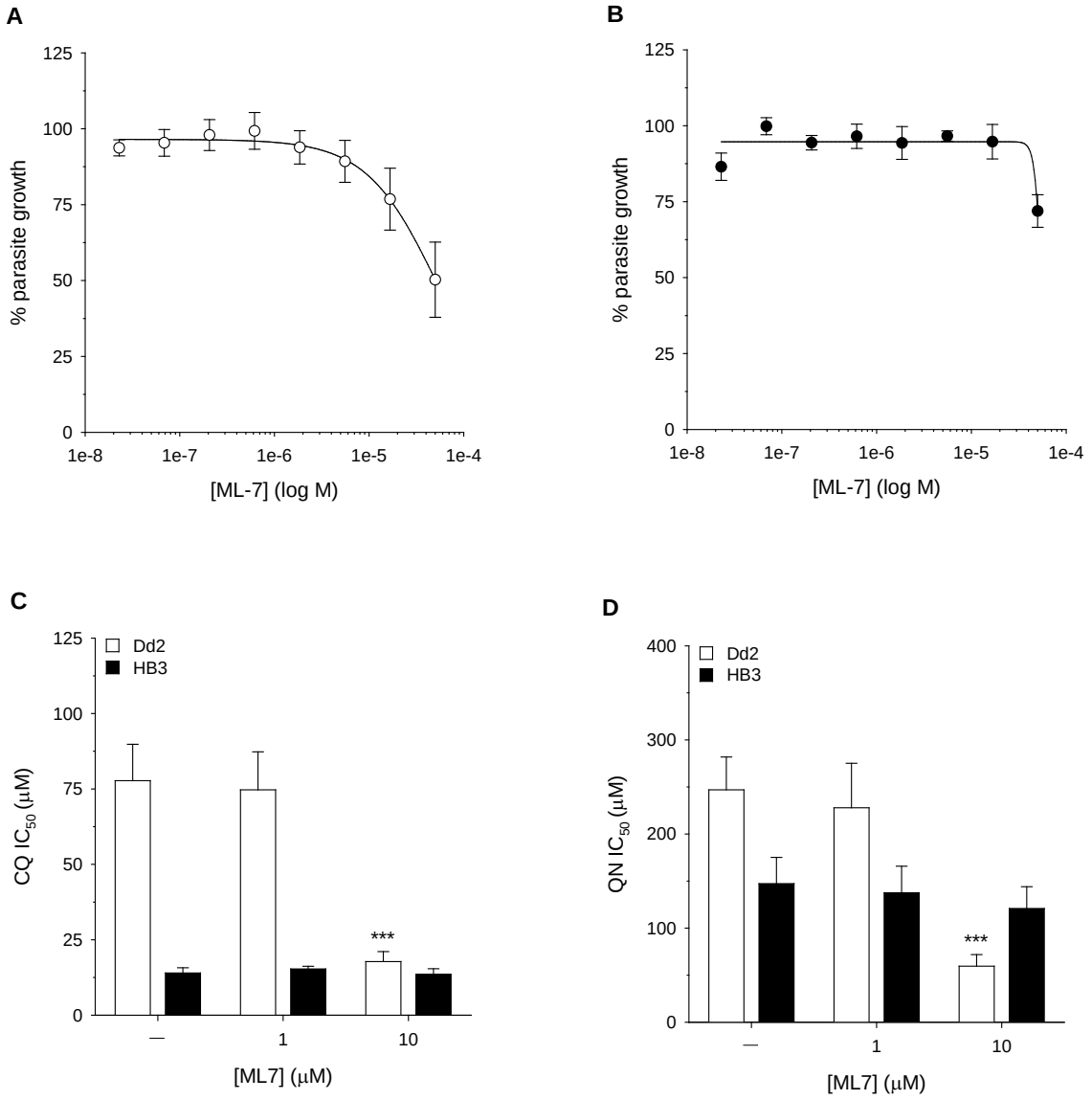


Figure 3.8. Effect of ML-7 on CQ and QN IC_{50} for Dd2 and HB3.

A. *ML-7 IC_{50} curve for Dd2. The data correspond to the percentage of growth at different concentrations of ML-7 after 72h of drug treatment. The values represent the mean ± SEM of six independent determinations.* ***B.*** *ML-7 IC_{50} curve for HB3. The data correspond to the percentage of growth at different concentrations of ML-7 after 72h of drug treatment. The values represent the mean ± SEM of five independent determinations.* ***C.*** *Chloroquine IC_{50} values for Dd2 and HB3 strains treated with different concentrations of ML-7 during the 72h period of incubation with CQ. The data represent the mean ± SEM of three to seven independent determinations. The difference between untreated Dd2 and Dd2 treated with 10 μM ML-7 was assessed using the ANOVA on Ranks test; p<0.001 (***). There was no significant difference between treated and untreated HB3.* ***D.*** *Quinine IC_{50} values for the Dd2 and HB3 strains treated with different concentrations of ML-7 during the 72h period of incubation with QN. The data represent the mean ± SEM of seven to twelve independent determinations. The difference between untreated Dd2 and Dd2 treated with 10 μM ML-7 was assessed using the ANOVA on Ranks test; p<0.001 (***). There was no significant difference between treated and untreated HB3.*

To study whether ML-7 could also reduce the concentration of CQ and QN necessary to inhibit the growth of CQ-resistant strains, the IC_{50} for CQ and QN of the Dd2 and HB3 strains was measured in the presence and absence of 1 μM and 10 μM of ML-7. As shown in Figure 3.8, the addition of 1 μM of ML-7 had no effect on CQ and QN IC_{50} values for both HB3 and Dd2 strains, but the addition of 10 μM of ML-7 caused a significant decrease on the CQ and QN IC_{50} values of Dd2, whereas no effect was observed for the HB3 strain.

To follow up on this result, a screen of 12 different ML-7 analogs was performed in order to find a compound with properties similar to ML-7 but a higher effect on CQ accumulation. The compounds were selected by the 4SC company in a similarity search using the core structure of ML-7 as a query (the compound structures can be found in appendix IV). The effects on CQ accumulation of each compound were analyzed at three different concentrations and compared with the accumulation in untreated parasites (1x accumulation).

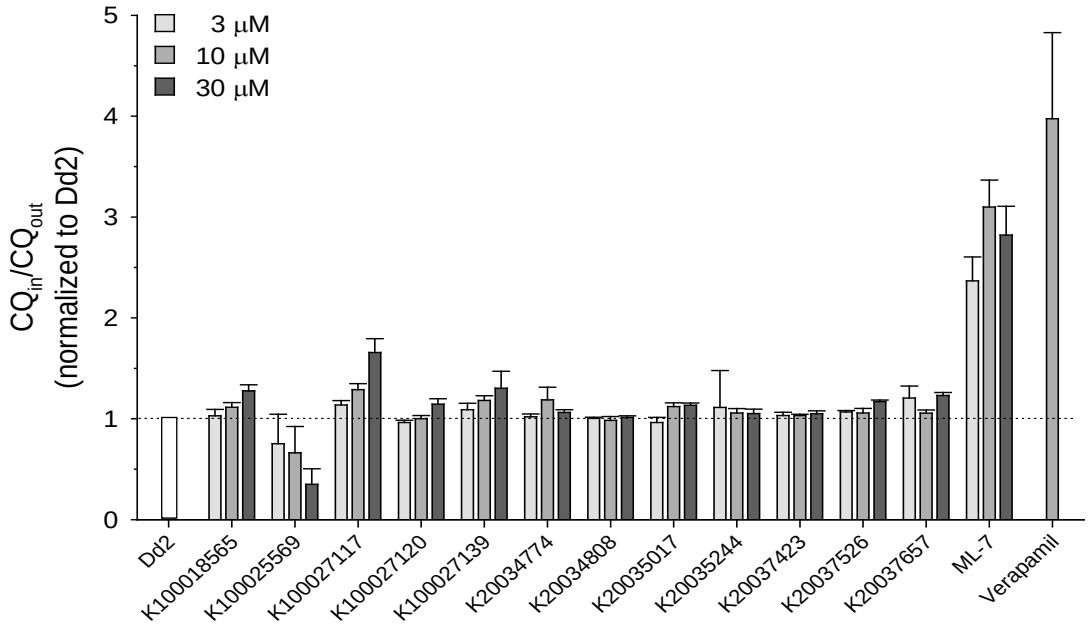


Figure 3.9. Screen of ML-7 analogs with an effect on CQ accumulation in P. falciparum.

Chloroquine accumulation of Dd2 treated with different ML-7 analogs at the 5 minute time point. Three different concentrations of each compound were used in the assay 3, 10 and 30 μM. The accumulation data are normalized to the CQ accumulation in untreated Dd2. The data represent the mean ± SEM of at least three independent determinations.

As shown in Figure 3.9, the parasites treated with ML-7 accumulated 3 times more CQ than the untreated parasites, whereas the parasites treated with the most active of the

ML-7 analogs only accumulated 1.65 times more CQ than the untreated control. Therefore, no better inhibitor than ML-7 was identified in this screen.

In a different approach, *Xenopus laevis* oocytes were used to assess the effect of ML-7 on PfCRT transport activity. *X. laevis* oocytes have previously been used to study the effect of resistance-reversing agents (Martin R et al., 2009). $PfCRT^{Dd2}$-expressing oocytes and water-injected control oocytes were incubated in acidic medium (pH = 6.0) with 10 µM of unlabeled CQ and trace amounts of [^{3}H]-CQ for 60 min, in presence or absence of ML-7.

As shown in Figure 3.10, a decrease in PfCRT-mediated CQ transport was observed when oocytes were treated with increasing concentrations of ML-7.

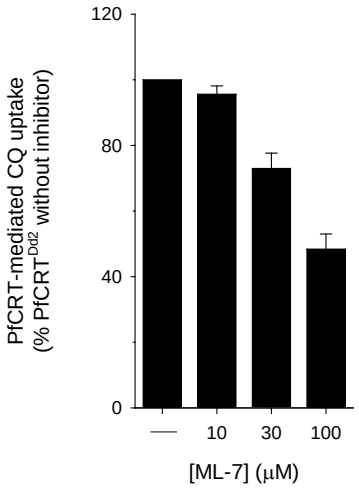


Figure 3.10. ML-7 influences the PfCRT-mediated CQ uptake in X. laevis oocytes.

PfCRT-mediated [^{3}H]-CQ uptake in X. laevis oocytes treated with different concentrations of ML-7. Three days post-injection oocytes were incubated for 60 min at 22°C in uptake buffer (pH 6.0) containing 10 µM CQ in the presence or absence of different concentrations of ML-7. The values correspond to the percentage of CQ uptake compared with untreated oocytes. The data represent the mean ± SEM of two to four independent determinations. Data generated together with Dr. Sebastiano Bellanca.

3.2.2. The downregulation of PfCK2 does not have an effect on CQ accumulation

Taking into account that 411 and 416 phosphorylation sites of PfCRT are consensus phosphorylation recognition sequences of CK2 and that the known PfCK2 inhibitor ML-7 increases the parasite's susceptibility towards CQ and QN, the role of PfCK2 in PfCRT regulation was further investigated. Two different strategies were followed: the

downregulation of PfCK2 and consequent phenotype analysis based on CQ accumulation experiments and the establishment of an *in vitro* phosphorylation assay.

A 3D7 strain with the CK2α endogenous locus tagged with hemagglutinin (HA) and the destabilization domain (DD) was kindly provided by the group of Prof. Alan Cowman. To evaluate the level of PfCK2α downregulation, the transgenic strain was cultured in the presence or absence of Shield-1 ligand over 24h. PfCK2α protein levels were quantified by Western blot for both conditions. As shown in Figure 3.11, the level of PfCK2α downregulation reached almost 90% after 24h in the absence of Shield-1. This outcome is in accordance with the data obtained by the group of Prof. Alan Cowman (unpublished data).

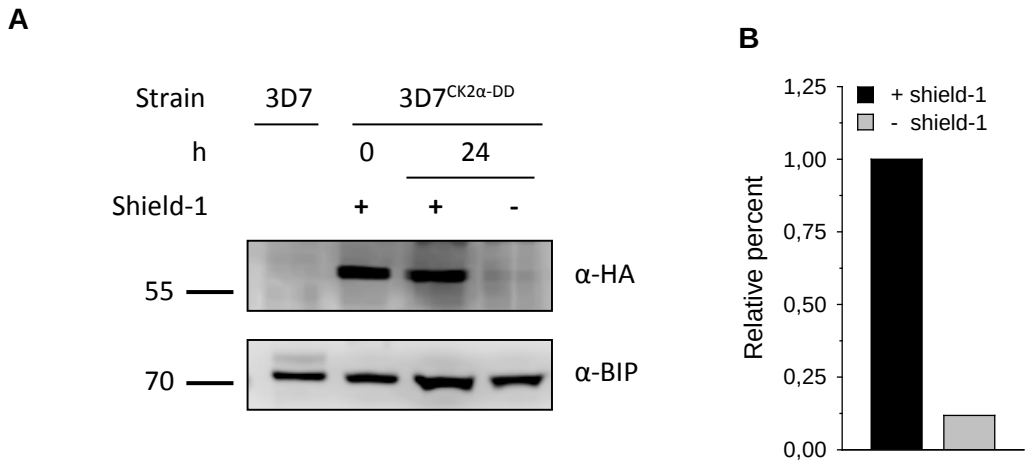


Figure 3.11. CK2α downregulation using the DD domain.

Young trophozoite synchronized cultures of the parental strain 3D7 and the transgenic strain 3D7$^{Ck2α-DD}$ were cultured in the presence or absence of 200 nM Shield-1 for 24h. Protein samples were collected at the 0 and 24 h time points. ***A.*** *Western blot using a mouse monoclonal anti-HA antibody and rabbit polyclonal anti-BIP antibodies. Expected molecular weights: PfCK2α-DD: 56 kDa; PfBIP: 72 kDa. Molecular weight markers at 55 and 70 kDa.* ***B.*** *Quantification of CK2α expression after 24h in the presence and absence of Shield-1 ligand. The quantification was performed using the Image studio Lite Ver 4.0 software.*

Taking into account that 3D7 is CQS, PfCRT^{Dd2} fused to GFP was episomally overexpressed in this strain in order to analyze the CQ accumulation phenotype when PfCK2α is downregulated. The 3D7^{CK2-DD} and the 3D7^{CK2-DD} + PfCRT^{Dd2}-GFP parasite lines were cultured in the presence or absence of Shield-1 ligand for 24 h and then CQ ratios within the infected erythrocytes and the extracellular medium were calculated at the 5 min time point.

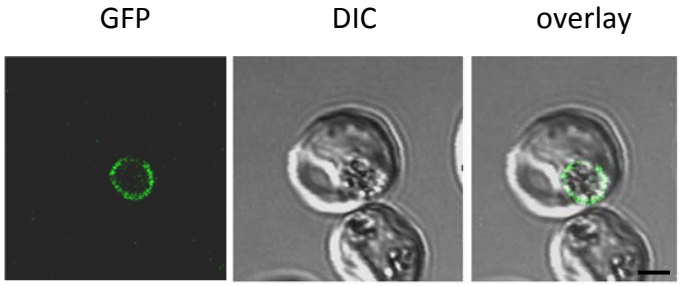


Figure 3.12. Episomal PfCRT^{Dd2}-GFP overexpression in the 3D7$^{CK2α-DD}$ strain.

Confocal live imagining of 3D7$^{CK2α-DD}$trophozoites expressing PfCRT^{Dd2}-GFP episomally. Left image, green channel; middle image, differential interference contrast (DIC); right image, overlay of both channels. Scale bar: 2 µm.

The strain 3D7 with the modified PfCK2α locus accumulated the same levels of CQ as the parental strain 3D7. When PfCRT^{Dd2} was overexpressed, the levels of CQ accumulation decreased, although not to the same levels as the resistant strain Dd2. In the absence of Shield-1, when PfCK2α was downregulated, the levels of CQ accumulation remained unaffected.

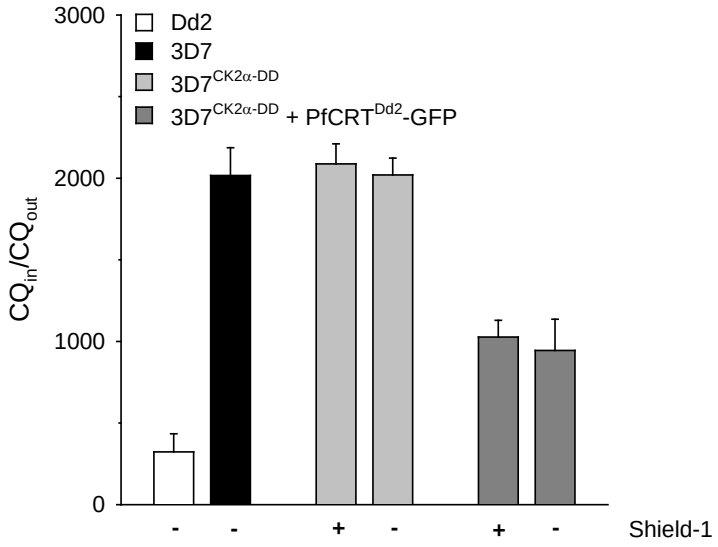


Figure 3.13. Effect of CK2α downregulation on CQ accumulation in P. falciparum.

Chloroquine accumulation in the Dd2, 3D7, 3D7$^{CK2α-DD}$ and 3D7$^{CK2α-DD}$ + PfCRT^{Dd2}-GFP strains in presence or absence of 200 nM Shield-1 at the 5 minutes time point. The data represent the mean ± SEM of three to four independent determinations. There was no significant difference when the 3D7$^{CK2α-DD}$ + PfCRT^{Dd2}-GFP strain was grown in absence or presence of Shield-1.

PfCK2α and the inactive mutant (PfCK2α^{K72M}) were overexpressed and purified from *E. coli* and preliminary phosphorylation assays were performed in order to test the activity of

the recombinant proteins. The results from the *in vitro* assay showed that the wild type protein had autocatalytic activity and that it was able to phosphorylate α-casein, while the mutant form was not active. The same amount of wild type and mutant enzyme was used on the assay, although the recombinant enzymes are not visible in the Coomassie-stained gel in Figure 3.14 due to the low amount of recombinant enzyme used in the assay. The concentration of both enzymes was quantified by Western blot before the kinase assay was performed.

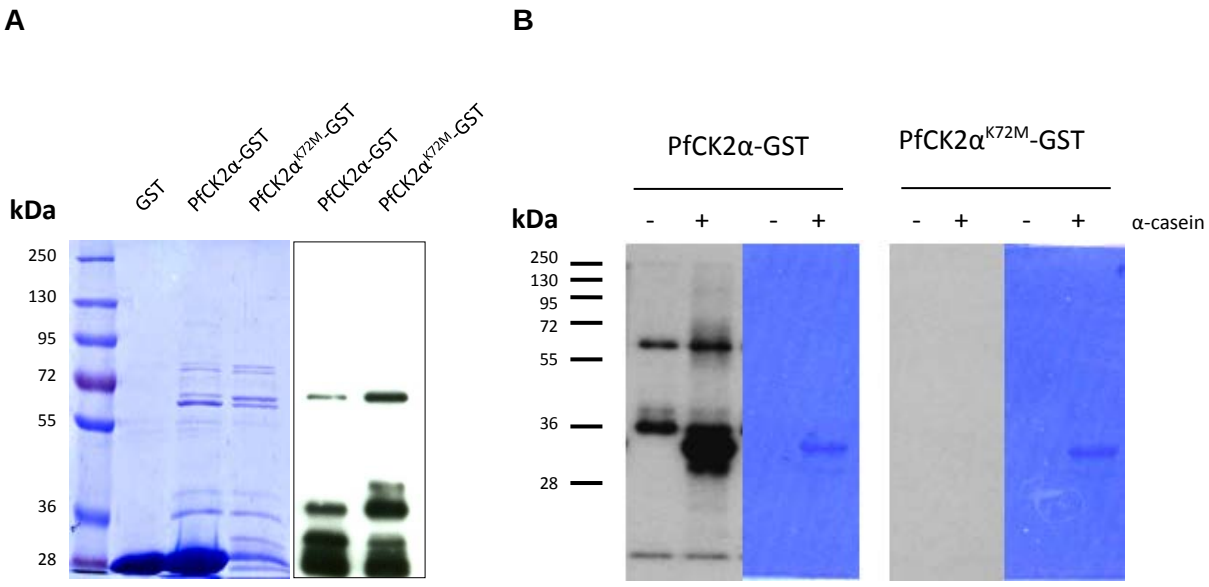


Figure 3.14. PfCK2α-GST and PfCK2α^{K72M}-GST kinase activity.

BL21 (DE3) cells expressing GST, PfCK2α-GST and PfCK2α^{K72M}-GST were induced with 0.1 mM IPTG for 20h. The recombinant proteins were then purified by affinity chromatography (glutathione beads) and used on the kinase assays. ***A.*** *Glutathione affinity purified GST, PfCK2α-GST and PfCK2α^{K72M}-GST. Left panel, Coomassie-stained SDS-PAGE; right panel, Western blot using a mouse monoclonal anti-GST antibody. Expected molecular weights: GST: 26 kDa; PfCK2α-GST and PfCK2α^{K72M}-GST: 66 kDa.* ***B.*** *PfCK2α-GST and PfCK2α^{K72M}-GST kinase assays. Autoradiograms on the left and Coomassie-stained SDS-PAGE on the right. As a substrate for the assays, 5 µg of α-casein or no substrate was used.*

In order to purify PfCRT to use it as a substrate for the phosphorylation assays, a Dd2 strain was transfected with a plasmid overexpressing PfCRT tagged with HA. As shown in Figure 3.15, PfCRT-HA exhibited the expected size and it was localized at the parasite's food vacuolar membrane.

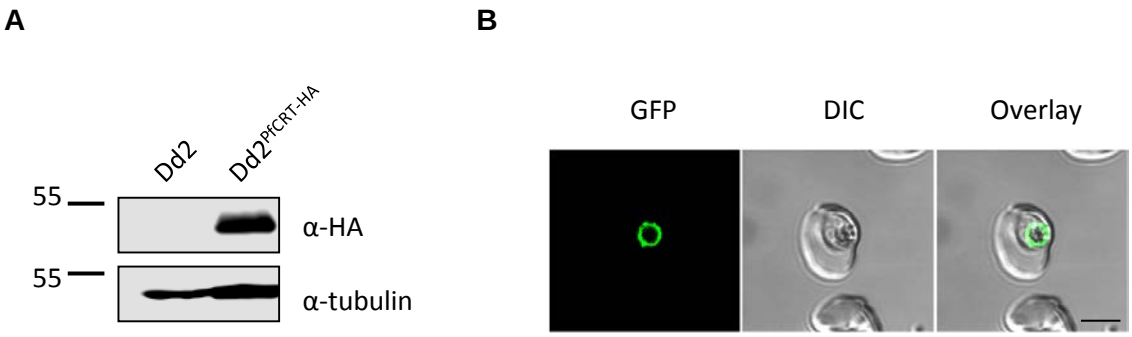


Figure 3.15. PfCRT-HA overexpression in P. falciparum.

Dd2 strain transfected with the episomal overexpression plasmid pAR-PfCRT^{Dd2}-HA. ***A.*** *Western blot using mouse monoclonal anti-HA and anti-tubulin antibodies. Expected molecular weights: PfCRT-HA: 51 kDa; PfTubulin: 50 kDa. Molecular weight marker at 55 kDa.* ***B.*** *Immunofluorescence of fixed Dd2 trophozoites overexpressing PfCRT^{Dd2}-HA using a mouse monoclonal anti-HA antibody. Left image, green channel; middle image, differential interference contrast (DIC); right image, overlay of both channels. Scale bar: 5µm.*

The overexpressed PfCRT-HA was immunoprecipitated and used as a substrate in *in vitro* phosphorylation assays to test the ability of PfCK2α to phosphorylate PfCRT. With the purpose of using it as a positive control of the assay, a *P. falciparum* extract was tested for its ability to phosphorylate α-casein and PfCRT-HA.

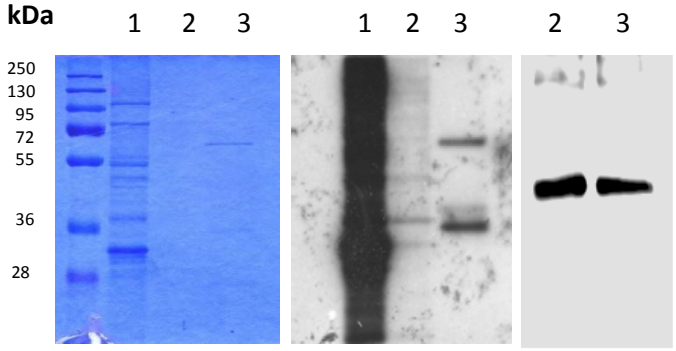


Figure 3.16. PfCRT-HA in vitro phosphorylation assay.

Kinase assays performed with the following reagents: lane 1, P. falciparum protein extract + α-casein; lane 2, P. falciparum protein extract + PfCRT-HA; lane 3, PfCK2α-GST + PfCRT-HA. Left panel, Coomassie-stained SDS-PAGE; middle panel, autoradiogram; right panel, Western blot using guinea pig polyclonal anti-PfCRT antibodies. The samples from the Coomassie-radiogram gel and the Western blot were prepared in the same reaction tube, but loaded into two different gels. Expected molecular weights: PfCRT-HA: 51 kDa; PfCK2α-GST: 66kDa.

No phosphorylation activity was detected for PfCRT-HA when using either CK2α or the *P. falciparum* extract. The immunoprecipitated PfCRT-HA protein was not visible on the Coomassie-stained gel, but it was detectable by Western blot, therefore if PfCRT-HA would have been phosphorylated it should have been possible to detect a clear signal on the autoradiogram. Due to the lack of a positive control, it was not possible to determine if PfCK2α phosphorylates PfCRT *in vitro*.

3.2.3. PF11_0488 characterization

In a previous Yeast Two Hybrid (Y2H) assay conducted in the lab by Anne Christin Roth, a serine/threonine kinase (PF11_0488 - PF3D7_1148000) was identified as an interaction partner of the C-terminal domain of PfCRT. A second Y2H assay was performed in order to further validate this result.

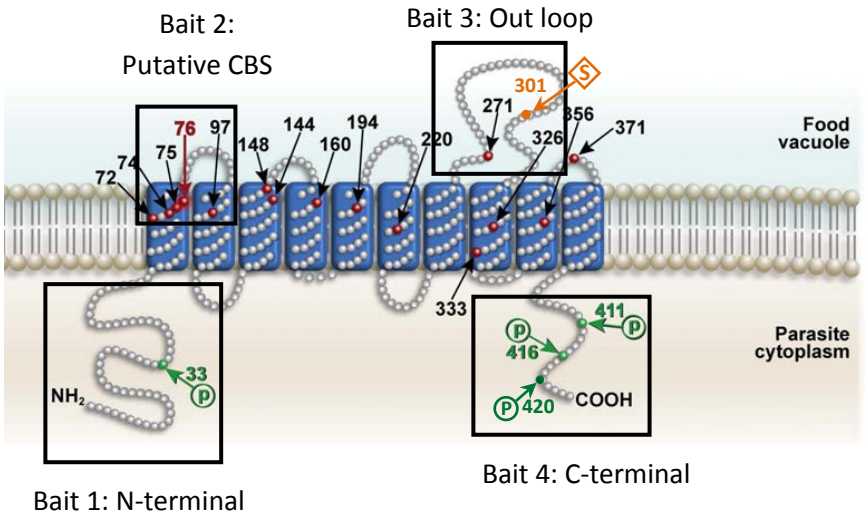


Figure 3.17. Y2H assay bait sequences.

Position of the bait sequences that were used in the Y2H assay in a PfCRT topological model. Black arrows point to polymorphic residues; the red arrow point to residue K76, a key residue in chloroquine resistance; green arrows point to residues that can be phosphorylated and the orange arrow point to the residue 301 that can be S-palmitoylated. Figure adapted from Sanchez et al. 2010.

In both assays, a *P. falciparum* cDNA library was used as prey and four different domains of PfCRT as bait: the N-terminal domain, a putative calmodulin binding site (CBS), the out loop and the C-terminal domain (fig. 3.17). Table 1 shows the summary of all the interaction partners of PfCRT found in the two assay replicates.

Bait	Prey
N-terminal	PF3D7_0623100: Coronin binding protein, putative
	PF3D7_0707300: Rhoptry-associated membrane antigen (RAMA)(2x)
	PF3D7_1024800: Conserved Plasmodium protein, unknown function
	PF3D7_1244800: Cytoplasmic translation machinery associated protein, putative
Putative CBS	PF3D7_0609000: Conserved Plasmodium protein, unknown function
	PF3D7_1133800: RNA (uracil-5-) methyltransferase, putative
	PF3D7_1207800: Conserved Plasmodium protein, unknown function
OUT loop	PF3D7_0106900: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, putative (IspD)
	PF3D7_0210600: Conserved Plasmodium protein, unknown function
	PF3D7_0907000: Conserved Plasmodium protein, unknown function
	PF3D7_0919800: TLD domain containing protein
	PF3D7_1033200: Early transcribed membrane protein 10.2 (ETRAMP10.2)
	PF3D7_1108600: Endoplasmic reticulum-resident calcium binding protein (ERC)
	PF3D7_1233600: Asparagine and aspartate rich protein 1 (AARP1)
	PF3D7_1324800: Dihydrofolate synthase/ folylpolyglutamate synthase (DHFS-FPGS)
	PF3D7_1425200: Enoyl-CoA hydratase, putative
C-terminal	PF3D7_0220000: Liver stage antigen 3 (LSA3)
	PF3D7_0406500: Conserved Plasmodium protein, unknown function
	PF3D7_1148000: Serine/ threonine protein kinase, putative

Table 3.1. Putative interaction partners of PfCRT.

Genes identified from a P. falciparum cDNA library as preys in two independent Y2H assays using four different sequences from PfCRT as baits. In black, preys found in the first assay (performed by Anne Christin Roth and Dr. Cecilia Sanchez) and in grey, preys found in the second assay. The PfCRT bait sequences and the exact sequences identified on the second assay can be found in appendix V.

There was no overlapping between the proteins identified in the two assays. Despite the fact that the interaction between PF11_0488 and PfCRT was not confirmed in the second assay, this kinase, which has been classified as an orphan kinase and described as essential for asexual growth in *P. falciparum*, was further characterized (Solyakov et al., 2011).

The cloning of the full length PF11_0488 sequence into the *P. falciparum* expression vector pARL1a+ was unsuccessful. Therefore, only the C-terminal fragment containing the sequences that code for the ATP binding region and the Ser/Thr protein kinase active site predicted by *Prosite* was cloned into the overexpression vector fused to a GFP tag. When PF11_0488$^{C\text{-terminal}}$ was overexpressed fused only to GFP, no expression was detected, therefore it was cloned fused to a GFP-CAD tag in order to control the expression levels and

avoid possible overexpression toxic effects. CAD is a conditional aggregation domain that aggregates in absence of Shield-1 (Rivera et al., 2000; Saridaki et al., 2008).

PF11_0488$^{C\text{-terminal}}$-GFP-CAD did not aggregate in the absence of the ligand, conversely showed a cytosolic localization (fig. 3.18). The expected size of the protein was confirmed by Western blot.

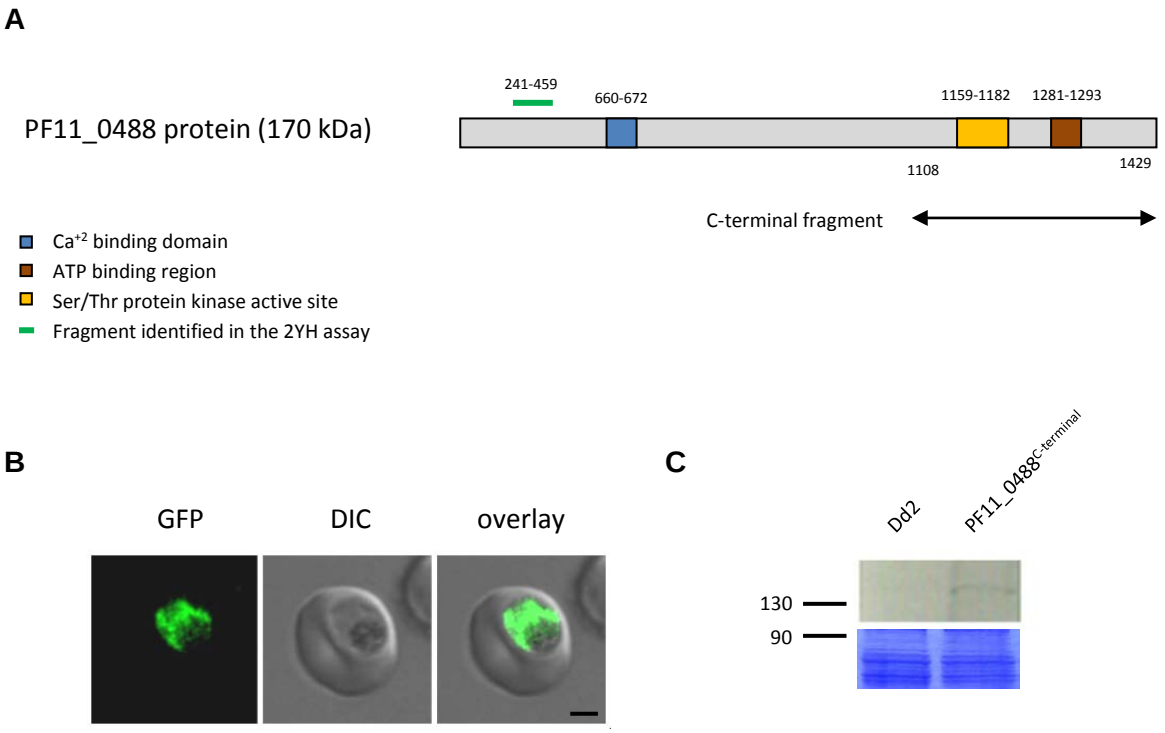


Figure 3.18. PF11_0488$^{C\text{-terminal}}$ -GFP-CAD overexpression in P. falciparum.

A. *Schematic representation of the PF11_0488 protein. The PF11_0488 domains predicted by Prosite are represented by different colors. The numbers indicate aminoacid positions. The length of the C-terminal fragment that was episomally overexpressed in P. falciparum and expressed and purified from E. coli is indicated by the black arrow. The fragment identified in the Y2H screening is indicated by the green line.* ***B.*** *Confocal live imaging of Dd2 trophozoites expressing PF11_0488$^{C\text{-terminal}}$-GFP-CAD episomally. Left image, green channel; middle image, differential interference contrast (DIC); right image, overlay of both channels. Scale bar: 2 µm.* ***C.*** *Upper panel, Western blot using a mouse monoclonal anti-GFP antibody. Expected molecular weight of PF11_0488 $^{C\text{-terminal}}$-GFP-CAD: 111 kDa. Down panel, gel stained with Coomassie blue as a loading control.*

These overexpressing parasites were analyzed for changes in CQ and QN accumulation as previously described. The overexpression of the C-terminal fragment of PF11_0488 reduced both the CQ_{in}/CQ_{out} and the QN_{in}/QN_{out} ratios. For CQ, the differences in accumulation between the parental and the transgenic strain were significant after 5 and 20 min of incubation with the drug, whereas for QN it was only significant after 20 min of

incubation (fig. 3.19). The accumulation values for the Dd2 + PF11_0488$^{C\text{-terminal}}$-GFP-CAD strain were the same in absence or presence of Shield-1 (not shown, data generated by Dr. Cecilia Sanchez).

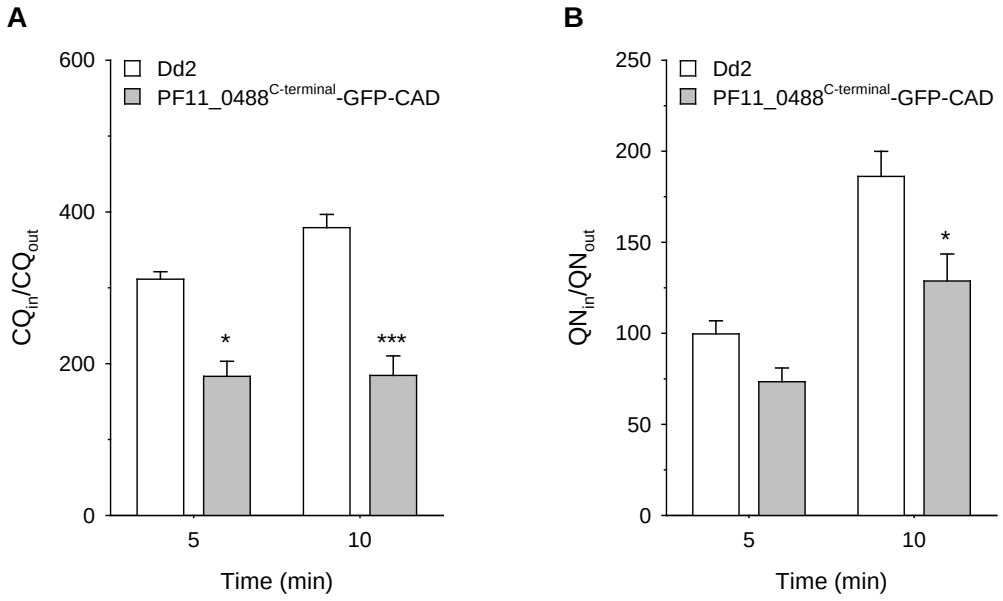


Figure 3.19. Effect of the PF11_0488$^{C\text{-terminal}}$-GFP-CAD overexpression on CQ and QN accumulation.

A. *Chloroquine accumulation ratios at the 5 and 20 minute time points in the transgenic strain Dd2 expressing PF11_0488$^{C\text{-terminal}}$-GFP-CAD episomally and in the parental strain Dd2.* ***B.*** *Quinine accumulation ratios at the 5 and 20 minute time points in the transgenic strain Dd2 expressing PF11_0488$^{C\text{-terminal}}$-GFP-CAD episomally and in the parental strain Dd2. The data represent the mean ± SEM of at least four independent determinations for the parental strain Dd2 and eleven independent determinations for the transgenic strain Dd2 expressing PF11_0488$^{C\text{-terminal}}$-GFP-CAD episomally. The differences between the parental strain Dd2 and the transgenic strain Dd2 expressing PF11_0488$^{C\text{-terminal}}$-GFP-CAD episomally were assessed using the T-test; p<0.05 (*); p<0.001 (***). Data generated together with Dr. Cecilia Sanchez.*

In order to confirm this result and taking into account the fact that PF11_0488 is considered essential during the erythrocytic asexual cycle of *P. falciparum* (Solyakov et al., 2011), the glmS ribozyme system (Prommana et al., 2013) was chosen to down-regulate this kinase instead of attempting a knock-out.

The CRISPR-Cas9 technology was used to introduce the glmS ribozyme into the 3'UTR of the PF11_0488 locus. Two different guide sequences were tested in this approach (see appendix II). As shown in Figure 3.20, the integration of the HA-glmS tag was successful in both cases.

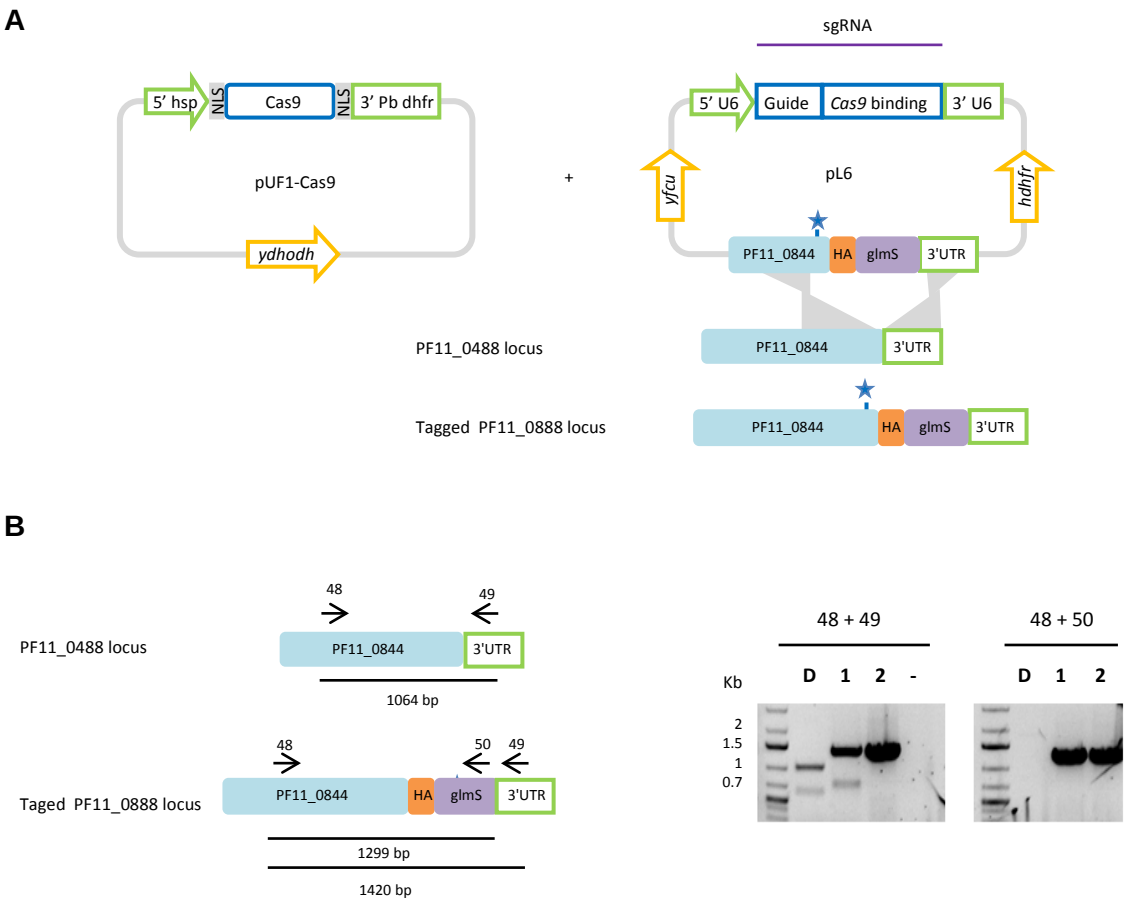


Figure 3.20. HA-glmS ribozyme tagging of PF11_0488 using the CRISPR-Cas9 system.

A. *Tagging strategy. The vector pUF1-Cas9 codes for the sequence of the Cas9 endonuclease flanked by nuclear localization signals (NLS). Its expression is regulated by the promoter region of the heat shock protein 86 (5' hsp) and the 3'UTR region of the P. berghei dhfr (3' Pb dhfr). The selection marker of the plasmid is the yeast dihydroorotate dehydrogenase gene (ydhodh). The pL6 plasmid contains the sgRNA-expression cassette. The expression of the sgRNA is regulated by the promoter and the 3'UTR region of the P. falciparum U6 snRNA polymerase III (5' U6). The selection marker of the plasmid is the human dihydrofolate reductase gene (hdhfr) and the negative selection marker is the bifunctional yeast cytosine deaminase and uridyl phosphoribosyl transferase (yfcu). A C-terminal PF11_0488 homology region of ~500 bp and a homology region of its 3'UTR, also of ~500 bp, were cloned before and after the HA-glmS tag respectively.The star indicates a shield mutation.* ***B.*** *Tag integration at the endogenous locus confirmed by PCR. Left, localization of the primers used in the PCR reaction and expected sizes of the PCR products for the endogenous and the tagged locus. Right, agarose gel of the PCR products obtained from the gDNA amplification of Dd2 (D) and Dd2 transfected with both pUF1-Cas9 and pL6-PF11_0488-HA-glmS-guide-1 (1) or pL6-PF11_0488-HA-glmS-guide-2 (2) plasmids.*

In order to quantify the downregulation level that is possible to achieve using this strategy, the transgenic strain was cultured in presence of increasing concentrations of glucosamine during 48 h. Before and after the treatment, PF11_0488-HA protein levels were quantified by Western blot.

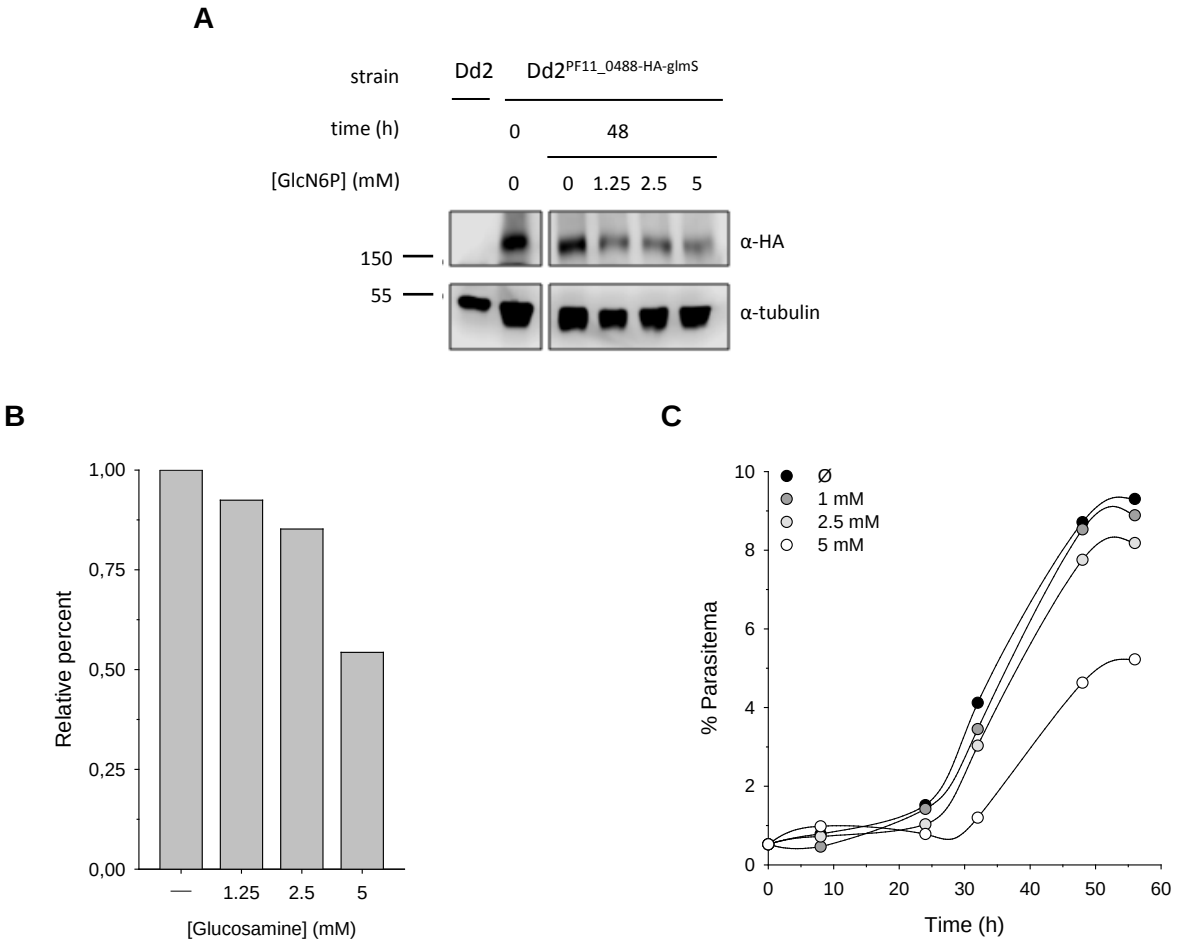


Figure 3.21. PF11_0488 downregulation using the glmS ribozyme.

Trophozoite synchronized cultures of the parental Dd2 and the transgenic Dd2$^{PF11_0844\text{-}HA\text{-}glmS}$ strains were treated with different concentrations of glucosamine over 48h. Protein samples were collected at time point 0 and 48 h after the treatment started. ***A.*** *Western blot using a mouse monoclonal anti-HA and anti-tubulin antibodies. Expected molecular weights: PF11_0488-HA: 175 kDa; PfTubulin: 50 kDa.* ***B.*** *Quantification of PF11_0488-HA expression at 48h, normalized to tubulin, at different concentrations of glucosamine.* ***C.*** *Dd2 growth curve in presence of different concentrations of glucosamine. Parasitemias over a time period of 56 h were determined by counting Giemsa-stained thin blood smears (~1000 RBC/slide).*

Only at a glucosamine concentration of 5 mM was the expression of the protein downregulated to 50%. However, this concentration of the compound, as seen in Figure 3.21, also affected the growth of the parental strain Dd2. Taking this result into account, the strain was not further characterized.

As a second approach, PF11_0488 was heterologously expressed in *E. coli* and the purified protein was tested for kinase activity. In order to purify the PF11_0488$^{C\text{-terminal}}$

fragment from *E. coli*, it was cloned into the expression vector pET28a and expressed fused to a 6xHis tag in RIL cells, which contain extra copies of the argU, ileY, and leuW tRNA genes. The cloning of the full-length sequence in this expression vector was also not achievable. Most of the protein aggregated in inclusion bodies in all the expression conditions tested. Nevertheless, it was possible to purify it from the soluble fraction after one affinity purification step, although the yield and the purity were not optimal. A second strategy followed in order to improve the yield and the purity of the protein was to purify it from inclusion bodies. The bacterial pellet was solubilized with 10% of the anionic detergent N-Lauroylsarcosine and then purified. This detergent has been used to solubilize functional proteins from inclusion bodies (IBs) with a 95% recovery efficacy (Tao et al., 2010).

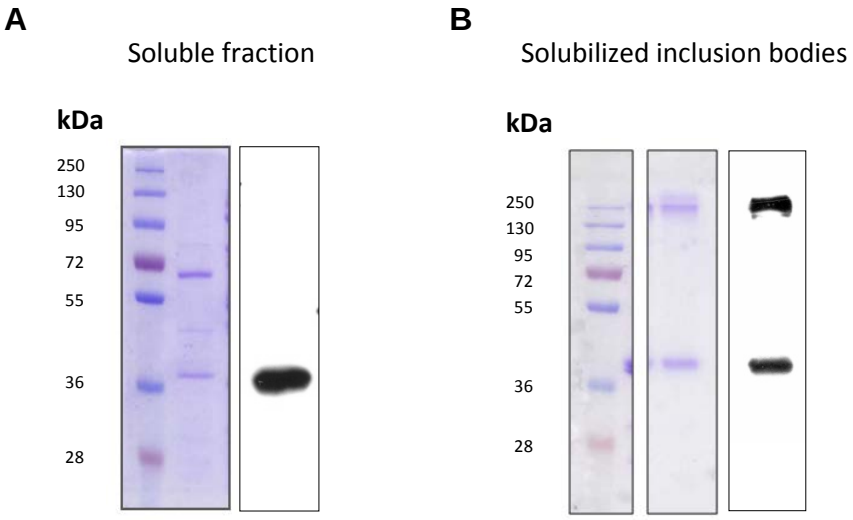


Figure 3.22.PF11_0488$^{C\text{-}terminal}$-his purification from E. coli.

RIL cells overexpressing the PF11_0488 C-terminal fragment fused to a 6xHis tag were induced with 0.05 mM IPTG for 4h at 25°C. ***A.*** *The soluble recombinant fraction was purified by affinity chromatography (Talon™ from Clontech). Left panel, protein gels stained with Coomassie blue; right panel, Western blot using a mouse monoclonal anti-His antibody.* ***B.*** *The inclusion bodies were first solubilized with 10% sarkosyl and then purified by affinity chromatography. Left panel, protein gels stained with Coomassie blue; right panel, Western blot using a mouse monoclonal anti-His antibody. The expected molecular weight for the PF11_0488 C-terminal-his protein is 39 kDa.*

Both purified proteins (soluble fraction and inclusion bodies solubilized with N-Lauroylsarcosine) were used to perform *in vitro* phosphorylation assays. The activity of both the soluble fraction and the solubilized inclusion bodies was tested against three ubiquitous substrates commonly used for *in vitro* phosphorylation assays: α-casein, myelin basic protein and histone H1. None of the substrates was phosphorylated by PF11_0488$^{C\text{-}terminal}$-his (data not shown).

As a second approach, the activity of the soluble fraction was tested against a *P. falciparum* extract that was heat inactivated, and against immunoprecipitated PfCRT-HA. Again, no phosphorylation activity was detected. As a positive control, the phosphorylation of α-casein by a *P. falciparum* protein extract is shown in Figure 3.23.

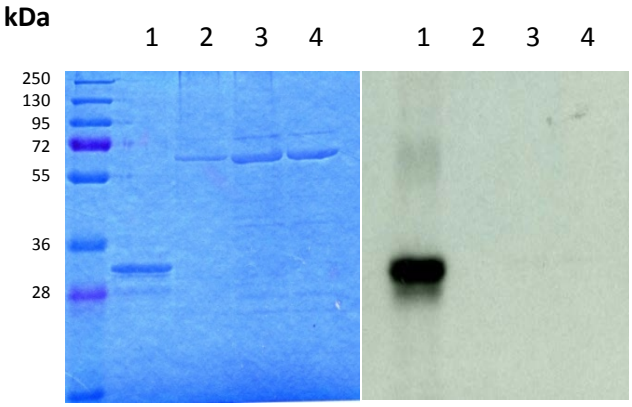


Figure 3.23. PF11_0488 in vitro phosphorylation assay.

Kinase assays performed with the following reagents: lane 1, P. falciparum protein extract + α-casein; lane 2, P. falciparum protein extract + PF11_0488$^{C\text{-}terminal}$-his soluble fraction (both heat inactivated); lane 3, P. falciparum protein extract (heat inactivated) + PF11_0488$^{C\text{-}terminal}$-his soluble fraction; lane 4, PF11_0488$^{C\text{-}terminal}$-his soluble fraction. Left panel, Coomassie-stained SDS-PAGE; right panel, autoradiogram.

3.2.4. PfCRT serine 33 modulates CQ and QN susceptibility and affects parasite's fitness

Genome editing in *P. falciparum* using the CRISPR-Cas9 system has been developed in recent years, opening up the possibility to introduce marker-free point mutations in endogenous genes with high efficiency (Ghorbal et al., 2014). This strategy was followed to mutate the residue S^{33} of PfCRT in the CQ-resistant Dd2 strain, from serine to alanine, in order to analyze the role of phosphorylation on PfCRT function. The $S^{33}A$ mutation was successfully introduced into the Dd2 genome and two clones, named B5 and B6, were isolated by limiting dilution. This strain will henceforth be referred to as $PfCRT^{S33A}$.

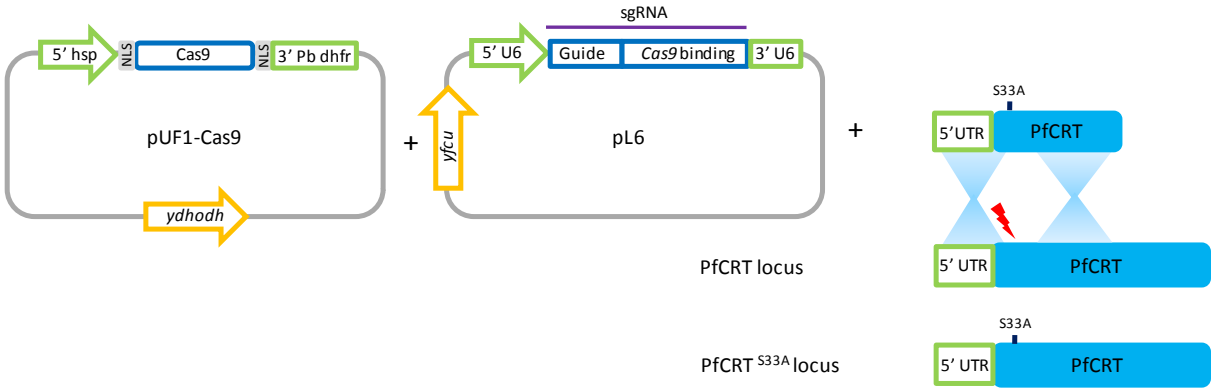


Figure 3.24. CRISPR-Cas9 strategy used to mutate PfCRT residue S^{33} from serine to alanine.

The vector pUF1-Cas9 codes for the sequence of the Cas9 endonuclease flanked by nuclear localization signals (NLS). Its expression is regulated by the promoter region of the heat shock protein 86 (5' hsp) and the 3'UTR region of the P. berghei dhfr (3' Pb dhfr). The selection marker of the plasmid is the yeast dihydroorotate dehydrogenase gene (ydhodh). The pL6 plasmid contains the sgRNA-expression cassette. The expression of the sgRNA is regulated by the promoter and the 3'UTR region of the P. falciparum U6 snRNA polymerase III (5' U6). The selection marker of the plasmid is the human dihydrofolate reductase gene (hdhfr) and the negative selection marker is the bifunctional yeast cytosine deaminase and uridyl phosphoribosyl transferase (yfcu). The homology region containing the $S^{33}A$ mutation (position -137 to 778) was transfected as a PCR product with modified ends (2 phosphorothioate-modified bases at the 5' end).

In order to assess the effect of this mutation on the sensitivity of the parasite towards CQ and QN, the half maximal inhibitory concentration (IC_{50}) was determined for both drugs. The chloroquine resistant strain (QRS) Dd2 and the chloroquine sensitive strain (CQS) HB3 were used as reference.

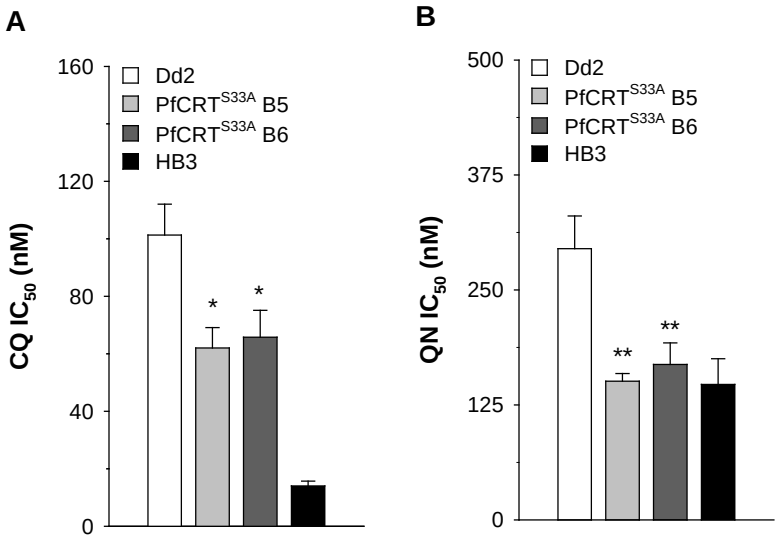


Figure 3.25. CQ and QN IC_{50} values for Dd2, PfCRT ^{S33A} and HB3.

A. *Chloroquine IC_{50} values in the mutant parasite lines, B5 and B6, in the parental strain Dd2 and in the CQS strain HB3 as a reference.* ***B.*** *Quinine IC_{50} values in the mutant parasite lines B5 and B6, in the parental strain Dd2 and in the CQS strain HB3 as a reference. The data represent the mean ± SEM of seven to eleven independent determinations per strain. The differences between the parental strain Dd2 and the transgenic strains PfCRT^{S33A} were assessed using the One Way ANOVA test; p<0.05 (*); p<0.01 (**).*

The CQ IC_{50} values decreased from 101 ± 11 nM in Dd2 to 62 ± 7 nM in B5 and 66 ± 9 nM in B6 when the residue S^{33} of PfCRT was mutated from serine to alanine. QN IC_{50} values were also reduced from 295 ± 36 nM in Dd2 to 151 ± 8 nM in B5 and 169 ± 23 nM in B6 when the mutant strains were compared to Dd2 (fig. 3.25).

A decrease in CQ and QN IC_{50} values is, in general, associated with a decrease in drug accumulation in the food vacuole. Therefore, CQ and QN accumulation was determined for the parental and the mutant strains as described previously. As shown in Figure 3.26, PfCRT^{S33A} clones accumulated the same level of CQ and QN as the parental strain Dd2 at any given time points.

To verify that the reduced CQ and QN susceptibility of the PfCRT^{S33A} strain was not due to a mislocalization of the transporter, an IFA using PfCRT antibodies was performed for this strain. The images in Figure 3.27.A confirmed that the mutant PfCRT was localized at the

embrane of the parasite's food vacuole as expected. This indicates that the protein is correctly localized despite carrying the $S^{33}A$ mutation.

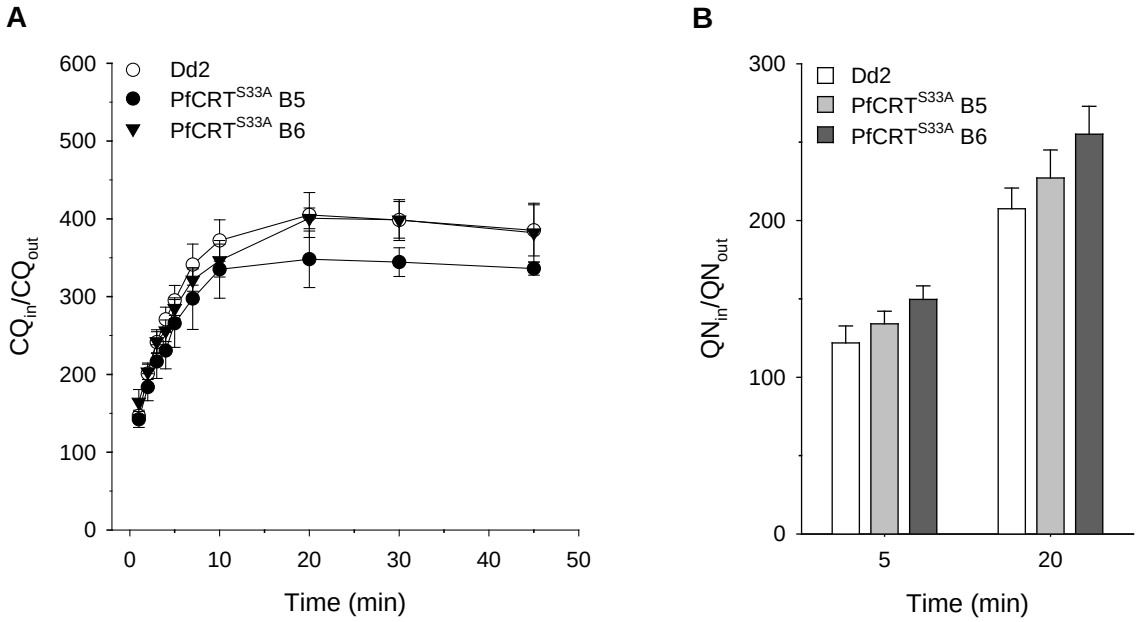


Figure 3.26. CQ and QN accumulation in Dd2 and PfCRT ^{S33A}.

A. *Time course of CQ accumulation ratios in the mutant parasite lines, B5 and B6, and in the parental strain Dd2. The data represent the mean ± SEM of five to six independent determinations per strain.* ***B.*** *QN accumulation ratios at the 5 and 20 minutes time point in the mutant parasite lines B5 and B6 and in the parental strain Dd2. The data represent the mean ± SEM of five independent determinations per strain. No significant difference was observed between the parental strain Dd2 and the transgenic strain PfCRT^{S33A}.*

Similarly, the stability of the mutant protein was assessed and compared to the wild type protein. The parental Dd2 strain and the two clones were treated with cycloheximide, a drug that inhibits protein synthesis (Schneider-Poetsch et al., 2010). Samples were collected at different time points after the beginning of the treatment and the protein levels over time were quantified by Western blot.

The endogenous control of the experiment was α-tubulin, which has shown to be stable after 8 hours of cycloheximide treatment in HeLa cells (Mi et al., 2009). As shown in Figure 3.27, both the wild type and the mutant proteins are equally stable.

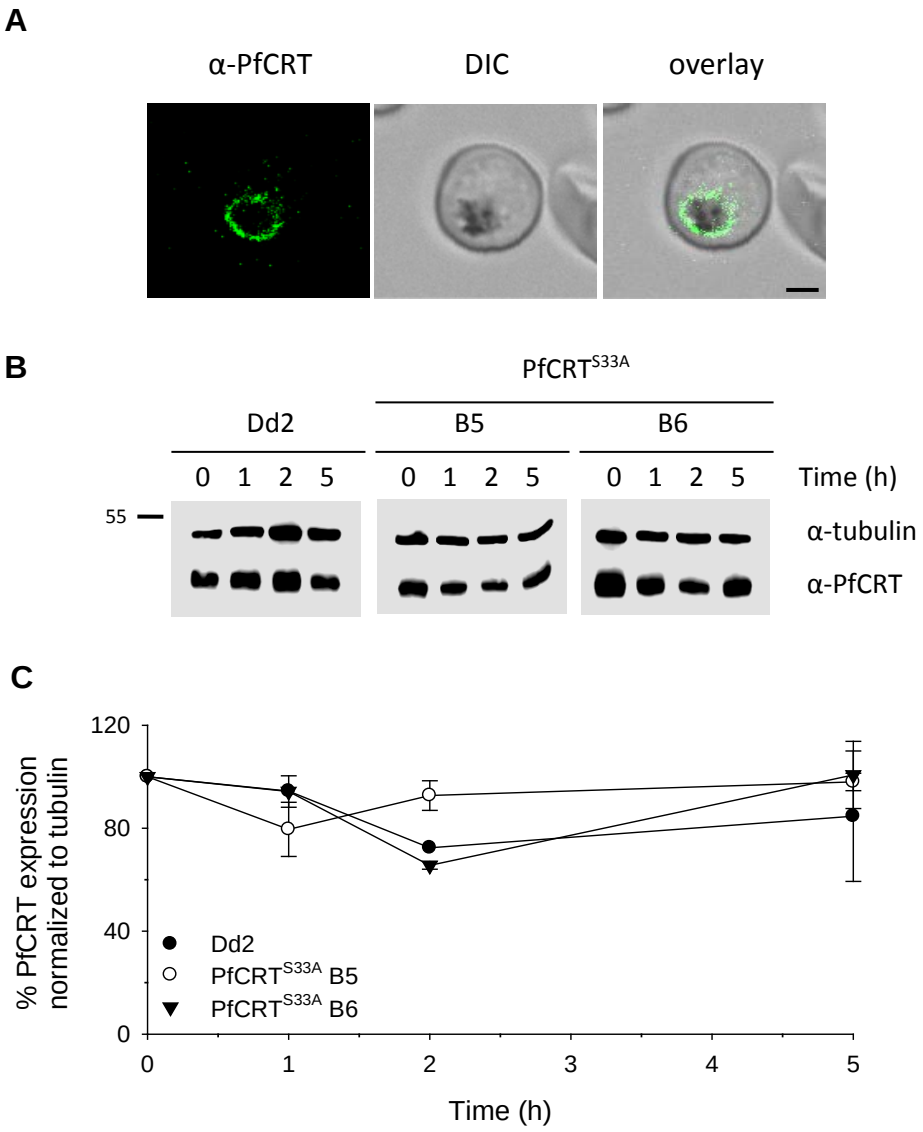


Figure 3.27. PfCRT^{S33A} localization and stability.

A. *Immunofluorescence of fixed Dd2 PfCRT^{S33A} (clone B6) trophozoites using mouse polyclonal anti-PfCRT antibodies. Left image, green channel; middle image, differential interference contrast (DIC); right image, overlay of both channels. Scale bar: 2 µm.* ***B.*** *A synchronized trophozoite culture of Dd2 and the transgenic strains PfCRT^{S33A} clones B5 and B6, were treated with 50 µg/ml of cycloheximide. Protein samples were collected at 0h, 1h, 2h and 5h after the treatment started. Western blot using mouse polyclonal anti-PfCRT antibodies and a mouse monoclonal anti-tubulin antibody. Expected molecular weights: PfTubulin: 50 kDa; PfCRT: 48 kDa. Molecular weight marker at 55 kDa.* ***C.*** *Quantification of PfCRT expression normalized to tubulin with the Image Studio Lite Ver 4.0 software. The data represent the mean ± SEM of two independent Western blot quantifications. No significant difference was observed between the parental strain Dd2 and the transgenic strain PfCRT^{S33A}.*

It has been previously shown that different *pfcrt* alleles confer different fitness characteristics to the parasite (Mita et al., 2004; Petersen et al., 2015). In order to test if the $S^{33}A$ mutation conferred a growth advantage or disadvantage in the presence and absence of CQ, the growth of both strains was compared by means of a fitness experiment.

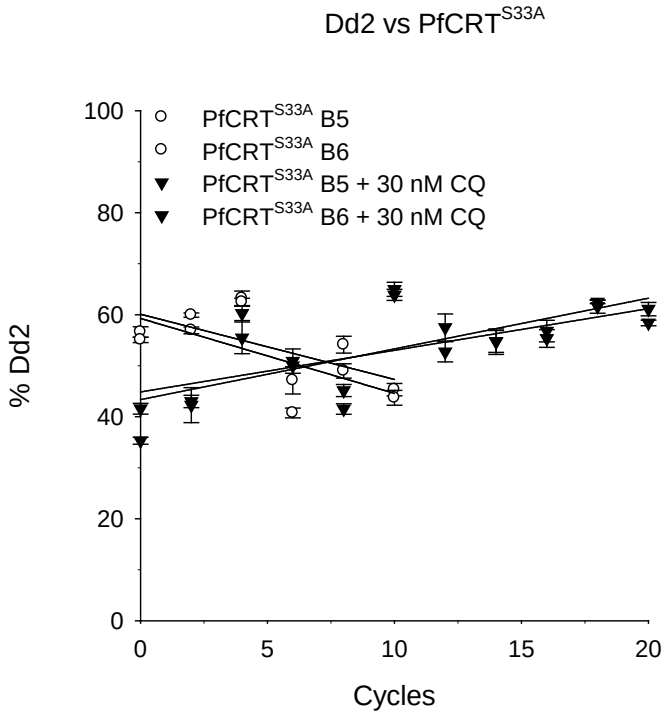


Figure 3.28. Effect of $PfCRT^{S33A}$ on parasite fitness.

Fitness assay. Mixed cultures at ~50% ratio between Dd2 and the mutant strain $PfCRT^{S33A}$ were cultured in presence or absence of 30 nM CQ. The allelic proportions were measured by pyrosequencing during 10 cycles in absence of the drug or during 20 cycles in the presence of CQ. The proportion of Dd2 in the cultures over time is represented on the Y axis. The data represent the mean ± SEM of three independent mixed cultures for each clonal line.

The parental Dd2 and the mutant $PfCRT^{S33A}$ strains were mixed together and kept in culture over 10 cycles. Every two cycles, samples of 3 replicates were analyzed by pyrosequencing to determine the proportion of each strain in the culture. The same experiment was performed in presence of 30 nM of CQ for 20 cycles. In the absence of drug pressure, the percentage of Dd2 in the culture decreased over time, indicating that the $PfCRT^{S33A}$ clones had better fitness than Dd2. Conversely, under drug pressure, the percentage of Dd2 in the cultures increased over time, suggesting that the $S^{33}A$ mutants are more sensitive to CQ than Dd2.

4. Discussion

4.1. PFE0825w characterization in *X. laevis* oocytes

The *Pfe0825w* gene is transcribed into three different mRNA variants. Variant 0 is the splice variant predicted *in silico* by PlasmoDB, but, as experimentally proven for this particular gene, often these predictions are inaccurate or incomplete. Particularly, the analysis of a *P. falciparum* cDNA library revealed that more than 20% of the investigated genes exhibited splicing events that were not correctly predicted (Lu et al., 2007).

Different mRNA variants were detected in different parasite strains. The fact that variant 0 was not detected in 7G8 and GB4 was probably due to differences in the methodology used to synthesize the cDNA. Asynchronous cultures were used to isolate the cDNA from 3D7, whereas 7G8 and GB4 cDNA was isolated from trophozoite synchronized cultures. *Pfe0825w* transcriptional profile data (Penarete-Vargas et al., unpublished) showed that variant 0 in asexual blood stages is the lowest transcribed variant. Therefore, the detection of variant 0 could have been overlooked in the 7G8 and GB4 strains.

Pfe0825w alternative splicing (AS) produces premature stop codons. The alternative transcripts can be targeted to nonsense mediated mRNA decay and therefore, the role of AS in this case could be associated with expression regulation (reviewed by Lykke-Andersen and Jensen, 2015). On the other hand, three protein isoforms with alternative start codons can be translated from the different splice variants. In *Plasmodium*, there are some examples where different isoforms of the same gene have different localizations and, potentially, different functions. AS generates two isoforms of MAEBL, an essential protein for merozoite invasion of erythrocytes and for sporozoite invasion of mosquito salivary glands, one of which is soluble and the other is membrane-bound (Preiser et al., 2004). The *cysRS* gene, cysteinyl tRNA synthetase, is also alternatively spliced and dually targeted to the apicoplast and the cytosol (Pham et al., 2014). Whether the different PFE0825w isoforms have different localizations or functions remains to be investigated. However, taking into account that the differences in the N-terminal sequence correlate with a different number of predicted transmembrane domains (TM) using the TMHMM server 2.0 (10 TM for variants 0 and 2 and 8 TM for variant 1), it is reasonable to speculate that the different isoforms have different biochemical properties.

The three mRNA variants were injected into *X. laevis* oocytes but only variants 0 and 1 were expressed and targeted to the oocyte oolemma. The fact that the sequence of the first 16 aminoacids in variant 2 was not codon optimized could explain why it was not possible to detect the expression of this protein isoform.

No differences in uptake between PFE0825w-expressing oocytes and water-injected oocytes were observed for any of the compounds tested in this study. The uptake conditions as well as the substrate concentrations used in the experiments were similar to the ones used to characterize other organic cation transporters in *X. laevis* oocytes (Grundemann et al., 1994; Kakehi et al., 2002; Okuda and Haga, 2000). For example, choline is transported into the parasite with an apparent K_m of 25-80 µM (Biagini et al., 2004; Lehane et al., 2004) and its physiological concentration in plasma is around 10 µM (Ozarda Ilcol et al., 2002), therefore the highest choline concentration (150 µM) used in the assays should have been sufficient to observe uptake. For the T3 compound, as it had never been used before in *X. laevis* uptakes, a concentration of 200 µM was chosen, consistent with the range of concentrations used in parasite uptakes (Wein et al., 2012).

Experiments carried out in Prof. Vial's group pointed to the predicted function of PFE0825w as albitiazolium and choline transporter (Penarete-Vargas et al., unplublished), thus the lack of transport activity in the oocyte system might be due to the limitations of this heterologous system. However, studies performed to discover interaction partners of this compound failed to identify PFE0825w as an albitiazolium target (Penarete-Vargas et al., 2014).

Considering that the expression of variants 0 and 1 at the oocyte membrane was confirmed by immunofluorescence, the lack of expression or the mistargeting of the transporter to another compartment are not problematic areas. At the parasite, the transporter is localized at the plasma membrane with the substrate binding site facing the parasitophorous vacuole (Penarete-Vargas et al., unpublished). Still, the topology of the transporter at the oocyte membrane is not a concern, because if the topology would be inverted in the oocyte, a decrease in accumulation would be expected. This is the case of the PfMDR1 transporter, where PfMDR1-expressing oocytes accumulate less vinblastine than water-injected oocytes (Sanchez et al., 2008a).

On the other hand, the transporter may be expressed at the oocyte oolemma, although incorrectly folded and therefore inactive. Moreover, the sequence of the transporter was mutated in order to remove the lysosomal trafficking motifs and these mutations may also affect the activity of the transporter.

Another possible explanation is that the transporter may not be active under the conditions used for the uptake experiments. Temperature can have a large effect on transport activity (Summers and Martin, 2010) and in the case of PFE0825w, it may be a critical factor. It has to be considered that it is not possible to carry out the experiments at the parasite's physiological temperature because it is much higher than that of the oocytes (37°C vs. 18°C).

It is also possible that the transporter requires association with a second protein to form a functional heterodimer, as is the case of the human sterol half-transporters ABCG5 and ABCG8 (Graf et al., 2004) or the *S. pneumonia* ABC multidrug efflux half-transporters PatA and PatB (Boncoeur et al., 2012).

Finally, different post translational modifications of the protein may take place in the parasite in comparison to the oocyte and this may have an effect on the activity and the substrate specificity of the transporter in the oocyte system. There are several examples of transporters whose activity and substrate specificity is regulated by post translational modifications such as the rOCT1 (Mehrens et al., 2000) and the hAQP2 (Moeller et al., 2011).

4.2. Analysis of the role of phosphorylation in the drug-resistance-mediating function of the chloroquine resistance transporter PfCRT

4.2.1. The kinase inhibitor ML-7 modulates CQ accumulation and CQ and QN susceptibility

The 411 and 416 phosphorylation sites of PfCRT match the consensus phosphorylation recognition sequence of casein kinase II (CK2), but the phosphorylation site at position 33 does not match any known kinase recognition sequence. For this reason, additionally to known CK2 inhibitors, a panel of inhibitors and activators targeting different classes of kinases and phosphatases was chosen to identify compounds with an effect on CQ accumulation.

From all the CK2 inhibitors tested, only ML-7 and rottlerin had an effect on CQ accumulation. Nevertheless, only three CK2 inhibitors from the panel have been shown to be active against the *P. falciparum* CK2 *in vitro*: ML-7, rottlerin and TBB (Holland et al., 2009). The fact that TBB did not show any effect on CQ accumulation may be due to the TBB concentration used in the assay; it might have been too low when compared with the concentration used in other cellular assays (Duncan et al., 2008; Ruzzene et al., 2002). On the other hand, quinalizarin has also been shown to be a potent inhibitor of PfCK2, although it was not tested in this study (Graciotti et al., 2014).

When analyzing phenotypes from kinase inhibitor treatments, careful interpretation of the results is pivotal since most of the compounds are not specific. All the inhibitors that had an effect on CQ accumulation target more than one kinase. ML-7, for example, is classified as a selective myosin light chain kinase inhibitor (MLCK) (Bain et al., 2003; Saitoh et al., 1987) but it also inhibits PfCK2α and hCK2α with an IC_{50} of 3-4 μM (Holland et al., 2009). H-89 inhibits PKA with an IC_{50} of 135 nM, but it also inhibits S6K1, MSK1, ROCK-II, PKBα, MAPKAP-K1b and MLCK (Davies et al., 2000; Umeda et al., 2008). W-7 is a calmodulin antagonist that inhibits the MLCK (Yamaki et al., 1979; Zimmer and Hofmann, 1984). Rottlerin, is also found to target multiple kinases *in vitro* and its use in cellular assays is questionable (Bain et al., 2003). Imipramine is a tricyclic antidepressant but its inhibitory effect on SRC and cyclic AMP-dependent protein kinases activity has also been reported (Ito et al., 1982; Nestler et al., 1989).

One possibility that needs to be ruled out is if the kinase inhibitors with an effect on CQ accumulation could inhibit the transport of CQ not by inhibiting a putative kinase, but by competing structurally with CQ to bind to PfCRT. Several compounds used in the assay share some chemical properties with CQ. For example, H-89 displays a quinoline chemotype; W-7, a naphthalene group with a chloride ligand, and imipramine, an azepane moiety in the tricyclic heterocycle. All those elements confer aromaticity to the compounds, a feature shared with CQ. In the case of ML-7, although it also exhibits this aromaticity, it is not probable that the compound competes with CQ because, an inhibition in QN transport should have also been observed. Furthermore, compounds with the same core structure as ML-7 did not show any effect on the accumulation assay. Uptake experiments of these radiolabelled compounds by *pfcrt*-injected oocytes would resolve this question.

Nevertheless, ML-7, W-7 and H-89 share a common target: the myosin light chain kinase (MLCK). Although the *P. falciparum* genome doesn't encode for any *mlck* gene, the parasite calcium-dependent protein kinase family includes members that share a high degree of homology with this kinase. The first hit on a BLAST search of the human myosin light chain kinase against the *P. falciparum* proteome is CDPK5 (e value = 8e-50). Furthermore, PbCRT has been identified in co-immunoprecipitation assays with PbCDPK4 in *P. berghei* gametocytes (personal communication from Mathieu Brochet). The hypothesis that CDPKs play a role on PfCRT phosphorylation is currently being investigated by other lab members.

When the CQS strain HB3 is treated with rottlerin, there is also a decrease in the amount of CQ that the strain accumulates. This indicates that the activity of this inhibitor is highly unspecific and is in agreement with the observation that rottlerin has multiple targets (Bain et al., 2003). Conversely, ML-7 only affects CQ accumulation in HB3 at high concentrations.

Taking into account the results from the screen of compounds that have a core structure similar to ML-7, it is clear that the increase in CQ accumulation is highly dependent on the specific structure of ML-7. Despite the high structural similarities between ML-7 and the compounds K20037526 and K100027117, they were not active in the assay. K20037526 displays both the diazepane ring and the naphthalene moieties but lacks the halogen. Conversely, K100027117 shows a halogen-substituted benzene, and a piperidine ring instead of the diazepane group. Furthermore, this result, together with the result from the first screen shows that CQ accumulation in Dd2 is only altered by a few specific compounds. There is no information available about the activity of the ML-7 analogs in any *in vitro* or *in vivo* assays, but the structure of the compounds can be found in appendix IV.

ML-7 not only affects CQ accumulation, but it also affects CQ and QN susceptibility. Although the Dd2 strain is slightly more sensitive to ML-7 than HB3, both strains show an IC_{50} for the compound higher than 50 µM, indicating that the compound does not have antiparasitic activity itself. When the Dd2 strain is treated with 10 µM of ML-7, its IC_{50} value for CQ decreases to sensitive levels. The same is true for QN, although ML-7 did not affect its accumulation. CQ and QN have been shown to be transported by PfCRT out of the food vacuole (Sanchez et al., 2005; Sanchez et al., 2008b) and the inhibition of PfCRT-mediated CQ transport by ML-7 could explain the phenotype observed for CQ but not for QN.

Therefore, it is reasonable to speculate that ML-7 is targeting different kinases or that the targeted kinase has multiple substrates.

QN accumulation does not always correlate with QN resistance as seen in the treatment of Dd2 with ML-7. The parasite lines $C4^{Dd2}$ and $C6^{7G8}$ are *pfcrt* allelic exchange mutants derived from GCO3, in which the endogenous PfCRT locus was replaced by the Dd2 or the 7G8 allele (Sidhu et al., 2002). These strains accumulate less QN than the parental one, and yet they are more sensitive to QN than the parental strain (Sanchez et al., 2008b). Furthermore, in the F1 progeny from a HB3xDd2 cross, parasites containing the PfCRT from Dd2 showed higher sensitivity towards QN than some parasites containing the HB3 allele (Ferdig et al., 2004). It has been suggested that QN has another target outside of the food vacuole and that when it is pumped out, it is accumulated in another organelle (Sanchez et al., 2008b). Along the same line, different *pfmdr1* alleles are not correlated with changes in QN accumulation but with changes in IC_{50} values, which led to the hypothesis that QN targets PfMDR1. The transport of Fluo-4 can be inhibited by QN in the parasites (Rohrbach et al., 2006) and in PfMDR1-expressing *X. laevis* oocytes (Sanchez et al., 2008a). Also the analysis of the F1 progeny of a GB4x7G8 cross showed no significant correlation between QN accumulation and QN IC_{50} values. In this study, QN accumulation was shown to be determined by PfCRT and QN resistance by PfMDR1. GB4 PfMDR1 conferred reduced resistance and GB4 PfCRT reduced accumulation. Conversely, 7G8 PfMDR1 conferred increased resistance and 7G8 PfCRT increased accumulation (Sanchez et al., 2011). Therefore, the observation that ML-7 has an effect only on QN IC_{50} but not on QN accumulation could be explained by the effect of this kinase inhibitor on PfMDR1 or on an alternative target of QN.

	Position within PfCRT									Position within PfMDR1				
Strain	72	74	75	76	220	271	326	356	371	86	184	1034	1042	1246
HB3	C	M	N	K	A	Q	N	I	R	N	F	S	D	D
7G8	S	M	N	T	S	Q	D	L	R	N	F	C	D	Y
GB4	C	I	E	T	S	E	N	I	I	Y	F	S	N	D
Dd2	C	I	E	T	S	E	S	T	I	Y	Y	S	N	D

Table 4.1. Polymorphisms within PfCRT and PfMDR1 in the strains HB3, 7G8, GB4 and Dd2.

To investigate whether ML-7 has a direct effect on PfCRT, PfMDR1 or on both transporters, it would be interesting to substitute the endogenous transporter locus of HB3 for the codon optimized versions of $PfCRT^{Dd2}$ or $PfMDR1^{Dd2}$, using the CRISPR-Cas9 system,

and then perform accumulation assays and determine the CQ IC_{50} of these strains in the presence of ML-7. Performing IC_{50} determination of other drugs not related to PfCRT, PfMDR1 and heme detoxification in presence of ML-7 would also support the hypothesis that the decrease of CQ and QN IC_{50} in the presence of ML-7 is not due to its toxic effects.

The effects of ML-7 on PfCRT-mediated CQ uptake in *X. laevis* oocytes have to be interpreted carefully. On the one hand, it is not possible to distinguish between competition of CQ and ML-7 for the binding pocket of PfCRT, and the effects of the inhibition of a putative oocyte kinase in CQ transport. Furthermore, it is not known whether PfCRT is phosphorylated or not when expressed in the oocyte. Attempts to identify the phosphorylation status of PfCRT in *X. laevis* oocytes have, so far, not been successful. On the other hand, both termini of the PfCRT sequence were modified in order to eliminate the endosomal-lysosomal trafficking motifs and these mutations are within the phosphorylation recognition sequences. The *in silico* prediction program PROSITE does not identify the residue S^{411} in the modified PfCRT sequence as a phosphorylation site.

4.2.2. The downregulation of PfCK2 does not have an effect on CQ accumulation

PfCK2 is formed by one catalytic subunit, PfCK2α, and two regulatory subunits, PfCK2β1 and PfCK2β2 (Holland et al., 2009). Each subunit is essential in blood stages and all localize dually to the cytosol and the nucleus. Interactions between the regulatory subunits and proteins from different metabolic pathways have been reported, with proteins involved in the chromatin assembly pathway being one of the most abundant (Dastidar et al., 2012).

PfCK2α downregulation mediated by the DD domain resulted in a protein expression decrease of 88%, comparable with previous reports (Armstrong and Goldberg, 2007).

The overexpression of $PfCRT^{Dd2}$-GFP in the $3D7^{CK2\alpha\text{-}DD}$ background led to CQ accumulation levels between Dd2 and 3D7 which are in agreement with similar values reported for the overexpression of $PfCRT^{Dd2}$-GFP in the HB3 background (Sanchez et al., 2014). When a protein is expressed from an episomal plasmid in *P. falciparum*, the percentage of cells expressing the transgene is lower compared to the same gene expressed from a chromosomal locus following integration. Also, the variance in expression levels observed between different parasites from the same culture is greater when the gene is expressed episomally (Adjalley et al., 2010; Nkrumah et al., 2006). Therefore, it was

expected that the level of CQ accumulation in the 3D7$^{CK2\alpha\text{-}DD}$ + pARL- PfCRT^{Dd2}-GFP strain would not reach the same level as reported for the Dd2 strain.

When the expression of PfCK2α is downregulated due to the absence of the stabilizing Shield-1 ligand, there are no changes in CQ accumulation. This result suggests that PfCK2α does not regulate PfCRT-mediated CQ transport. Nevertheless, it can not be ruled out that the remaining 12% of the enzyme is sufficient to regulate CQ transport, to the extent that no phenotypical changes can be observed, or that other kinases are complementing its function.

The outcome from the heterologous overexpression of PfCK2α-GST and PfCK2α^{K72M}-GST experiments and from the *in vitro* kinase assays is comparable to the previously reported results. PfCK2α-GST has autophosphorylation activity and is able to phosphorylate α-casein *in vitro*. The nature of the phosphorylated protein that runs at 36 kDa is unknown, but considering the fact that a signal is also detected in the Western blot against GST at the same molecular weight, it is probably a degradation product. The K72 residue is involved in the binding of ATP to the active site of the kinase, therefore the PfCK2α^{K72M}-GST mutant is inactive and can be used as a negative control (Holland et al., 2009).

In order to obtain PfCRT to use as a substrate in the kinase assay, the protein was tagged with a 3xHA tag and overexpressed in *P. falciparum*. The 3xHA tag was choosen due to its small size, compared with other tags, in order to reduce the possibility of a nonspecific phosphorylation of the tag. PfCRT-HA was not phosphorylated by PfCK2α-GST or by any protein from a *P. falciparum* extract in the *in vitro* assay. In the absence of a known kinase that phosphorylates PfCRT, a *P. falciparum* protein extract was used, under the assumption that one of the proteins in the extract could posphorylate PfCRT. The protein extract proved to contain active kinases that were able to phosphorylate α-casein as well as other proteins from the extract. The lack of PfCRT-HA phosphorylation may be due to at least two reasons. First, the low amount of PfCRT-HA used as a substrate in the assays compared with the amount of α-casein, although as it was possible to detect the same amount of protein used in the assay by Western blot, it is reasonable to assume that it should have also been possible to detect a signal in the autoradiogram. The second possibility is that because to extract PfCRT-HA from the membrane, anionic detergents had to be used and because PfCRT-HA was still bound to the sepharose beads when used in the assay, this could have an

efect on the PfCRT tertiary structure, preventing its recognition by the putative kinase. Without having a positive control of the assay, it is not possible to conclude whether or not PfCK2α phosphorylates PfCRT *in vitro*.

4.2.3. PF11_0488 characterization

To find interaction partners of membrane proteins is a challenging goal, particularly in the case of PfCRT. Co-immunoprecipitation experiments with PfCRT are very challenging because protein interactions are disrupted by the high concentration of anionic surfactants necessary to isolate the protein from the membrane. The use of the Y2H system, at least, allows interaction partners of the soluble fragments of a membrane protein to be identified. This was the chosen strategy to find PfCRT´s interacting partners. The two independent assays performed did not identify the same interacting proteins. This result is not particularly striking. Indeed, two large-scale assays which aimed to identify two-hybrid interactions in the entire yeast proteome, performed independently, had less than 30% overlap of positive interactions and, in general, only 12.5% of previously known interactions were identified (Ito et al., 2001). Recently, a new methodology called BioID (proximity-dependent biotin identification) which enables the identification of protein interactions in eukaryotic cells has been developed. This technique enables detection of protein interactions in their cellular environment by tagging the protein of interest with a biotin protein ligase. The proteins that are in close proximity to the tagged protein are, therefore, biotinylated and thus, can be identified (Roux et al., 2012). This technique has already been applied to study protein interaction in the protozoan parasite *Toxoplasma gondii* (Chen et al., 2015) but there are, as yet, no reports in *P. falciparum*. This would be a better approach to find interaction partners of PfCRT because the full length protein in its native conformation could be assayed in its cellular environment.

Apart from the putative serine/threonine protein kinase PF11_0488, the rhoptry-associated membrane antigen (RAMA) was also identified as a potential PfCRT-interacting protein. RAMA has been found to be significantly associated with the mutated form of PfCRT, although it seems to be part of the low variability region harboring PfCRT that segregates with it (Sanchez et al., 2014). Further experiments need to be done to confirm this interaction and unravel the molecular meaning of this association. Other preys found in the assay are related to different metabolic pathways that have, so far, not been linked with

PfCRT function, thus the confirmation of these interactions or a deeper analysis of these proteins was not a priority.

PF11_0488 is expressed at the trophozoite and early schizont stages (Ward et al., 2004) and its expression is highly correlated with the expression of PfCRT (Adjalley et al., 2015). It has been classified as an orphan kinase and it is essential for the asexual growth in *P. falciparum*. Attempts to knock out (KO) the gene were not successful but it was possible to tag it at the C-terminal, suggesting that the absence of integration of the KO vector was not caused by locus refractoriness to recombination, but rather due to the fact that the gene is essential during the asexual growth of the parasite (Solyakov et al., 2011). However, the ideal experiment would have been to disrupt the gene while complementing its function through episomal expression.

Taking into account the fact that *pfcrt* is suggested to be an essential gene (Waller et al., 2003) and that phosphorylation of PfCRT at the residue T^{416} is necessary for the correct localization of the transporter (Kuhn et al., 2010), it is reasonable to assume that the putative kinase that phosphorylates PfCRT would also be essential.

The cloning of the full length *PF11_0488* gene in different expression vectors was unsuccessful. Therefore, the C-terminal fragment that contains the coding sequences for the ATP-binding region and the Ser/Thr protein kinase active site predicted by PROSITE was used as an alternative to characterize this protein. All the InterPro domains annotated for this protein in the PlasmoDB database are also within this fragment, supporting the choice of this coding region. Furthermore, this is the most conserved region between *Plasmodium* species (see appendix II). It is not possible to predict if this protein fragment is catalytically active but its overexpression in *P. falciparum* showed an effect on CQ and QN accumulation. It has also been shown before, that the expression of a truncated form of an enzyme can retain its catalytic activity as in the case of the *P. falciparum* HECT ubiquitin-protein ligase (Sanchez et al., 2014). Truncated enzymes from different organisms have also been used in order to characterize and resolve the crystal structure of enzymes when the full length protein is poorly soluble (Hilden et al., 2000; Joucla et al., 2006).

When PF11_0488$^{C\text{-terminal}}$ was overexpressed fused only to GFP, no expression was detected by fluorescence microscopy. Conversely, when fused to GFP-CAD, a strong cytosolic signal was observed. It is possible that the PF11_0488$^{C\text{-terminal}}$-GFP overexpression was toxic for the parasite and that when the protein was fused to CAD, part of it aggregated (Rivera et

al., 2000; Saridaki et al., 2008), thereby decreasing the active concentration of the protein to non-toxic levels. Another possibility is that the GFP tag alone alters the conformation of the protein and makes it inactive, generating a dominant negative form of the protein, which causes parasite death upon overexpression.

The PF11_0488$^{C\text{-terminal}}$-GFP-CAD overexpression in the *P. falciparum* Dd2 strain decreased the levels of CQ and QN accumulation in the parasite. This indicates that PF11_0488 is somehow enhancing the transport of these two drugs out of the food vacuole. This is contrary to the effect observed when the Dd2 strain is treated with kinase inhibitors, leading to the general observation that phosphorylation inhibition leads to lower levels of CQ and QN accumulation. This effect is unrelated with PfCRT S^{33} phosphorylation since the substitution of this residue by alanine doesn't have an effect on CQ or QN accumulation. However, uptake experiments of a Dd2 strain which episomally overexpresses GFP-CAD would have helped to rule out any effect of the overexpression of the GFP-CAD tag itself. This is particularly a concern in this case as, the GFP-CAD tag is bigger than PF11_0488$^{C\text{-terminal}}$ itself, which could also affect the conformation of the protein fragment. Overexpression of PF11_0488$^{C\text{-terminal}}$-GFP-CAD and consequent uptake experiments in the HB3 strain would have also been a good control to reject the possibility of an unspecific effect. Nevertheless, overexpression of other proteins tagged with GFP showed no effect on CQ and QN accumulation (Sanchez et al., 2014).

The HA-glmS tagging of the endogenous *PF11_0488* was achievable using two different guide sequences. Conversely, it was not possible to introduce a GFP or a GFP-DD tag into the locus using the same homology regions and the same guide sequences. For guide 2, the recombination took place between the guide sequence and the GFP tag, so the double break was repaired by the introduction of the shield mutations but the tag was not inserted (data not shown). This outcome points to the fact that the GFP tag is interfering with the protein function, as already seen before in the episomal expression of PF11_0488$^{C\text{-terminal}}$.

It was not possible to downregulate PF11_0488 using the glmS system. Only when the parasites were treated with 5 mM of glucosamine, the expression of PF11_0488 decreased by 50%. However, this glucosamine concentration causes growing defects of the parental strain Dd2. Even higher concentrations of glucosamine (6-10 mM) have been used in other studies, although no significant decrease in parasitemia was found when the

parental strain 3D7 was treated with 5 mM of glucosamine (Prommana et al., 2013; Sleebs et al., 2014). Other members of the lab have also observed that 5 mM of glucosamine is a toxic concentration for the Dd2 strain therefore, this phenotype may be strain-specific. Currently, there are other strategies available to downregulate the expression of *P. falciparum* genes that could be applied to PF11_0488 such as the DD system (Armstrong and Goldberg, 2007). Another strategy that could be applied is to delete the gene using a conditional deletion system such as the diCre system (Collins et al., 2013a) which allows an efficient control of the recombination levels through the addition of the rapamycin ligand.

The recombinant expression of PF11_0488$^{C\text{-terminal}}$-his resulted in high production of inclusion bodies and a minor fraction of soluble protein. Both forms of the protein fragment were tested for activity in phosphorylation assays *in vitro*, but no activity was detected. The possibility that any of the substrates used in the assays was suitable for this kinase was ruled out by using an inactivated *P. falciparum* protein extract. Even so, the appropriate substrate could have been present in amounts below the detection level. It is possible that only the full length protein is functional and that the Ca^{+2}-binding domain is essential for its activity (fig. 3.18). Along the same line, PF11_0488 is phosphorylated at residues T669 and S680, suggesting that it could participate in a protein kinase cascade. These phosphorylations can also be crucial for the activity of the kinase. Nevertheless, the overexpression of PF11_0488$^{C\text{-terminal}}$-GFP-CAD in the *P. falciparum* Dd2 strain decreased the levels of CQ and QN accumulation, suggesting that the PF11_0488$^{C\text{-terminal}}$ fragment is active, although the effects of the GFP-CAD tag overexpression alone were not investigated.

4.2.4. PfCRT serine 33 modulates CQ and QN susceptibility and affects the parasite fitness

The mutation of the PfCRT residue S^{33} to alanine was achieved using the CRISPR-Cas9 system, which has recently been adapted to *P. falciparum* (Ghorbal et al., 2014). Alanine was chosen to substitute serine because it is structurally the closest amino acid. Attempts to mutate this position to aspartic acid and glutamic acid in order to rescue the S^{33}A phenotype were pursued but have so far been unsuccessful. Due to the lack of additional mutants, the phenotype that resulted from the S^{33}A mutation cannot be directly linked to absence of phosphorylation since the possibility that the S^{33}A mutation alters the structure of the transporter cannot be ruled out.

The PfCRT^{S33A} strains showed a significant decreased in the IC_{50} values for both CQ and QN, meaning that this mutation increases the susceptibility of the parasite towards these drugs. On the other hand, there were no differences in CQ or QN accumulation between the parental and the mutant strain. It has been shown that CQR strains accumulate lower levels of CQ compared to CQS strains (Fidock et al., 2000; Sidhu et al., 2002) but the analysis of the F1 progeny of a genetic cross between a CQR and a CQS strain showed that there is not always a correlation between the degree of susceptibility and the concentration of the drug in the digestive vacuole, suggesting that polymorphisms within transporters contribute to drug resistance through molecular mechanisms that have yet to be identified. It has been hypothesized that resistance associated with little change in drug accumulation could be explained by CQ blocking the transport of the physiological substrate of PfCRT, suggesting that PfCRT itself is also one of the CQ drug targets (Sanchez et al., 2011). The same result was obtained when an endogenous copy of PfCRT was substituted by different *pfcrt* alleles in the same genetic background (Petersen et al., 2015). In the same publication, different *pfcrt* alleles conferred different fitness properties, as also reported in this study.

Considering the fact that the localization and the stability of the mutant PfCRT^{S33A} are not affected, the differences in drug susceptibility and fitness between the mutant and the parental strain must be related to the function of PfCRT. Furthermore, taking into account the fact that the susceptibility of the mutant strain is not linked to CQ and QN transport out of the food vacuole (at least in a short term experiment), it is reasonable to assume that the $S^{33}A$ mutation affects the transport of the physiological substrate of PfCRT as previously hypothesized.

CQ and QN inhibit ferriprotoporphyrin IX (FP) crystallization (Fitch and Chou, 1997; Sullivan et al., 1996). On the one hand, CQ binds to monomeric FP and delays its detoxification (Chugh et al., 2013; Fitch, 1986). On the other hand, the exact mode of action of QN is still under discussion. Although at least 80% of the FP is converted to hemozoin at the trophozoite stage (Egan et al., 2002), the estimated concentration of free FP is about 0.1 mM (Loria et al., 1999). One possible explanation for the phenotype observed in the PfCRT^{S33A} mutants is that PfCRT transports FP out of the food vacuole to supply the parasite with Fe^{+3}. FP transporters have been characterized in some organisms, including the pathogen bacterium *Yersinia pestis* (Woo et al., 2012) and the nitrogen-fixing bacterium *Sinorhizobium meliloti* (Cuiv et al., 2008).

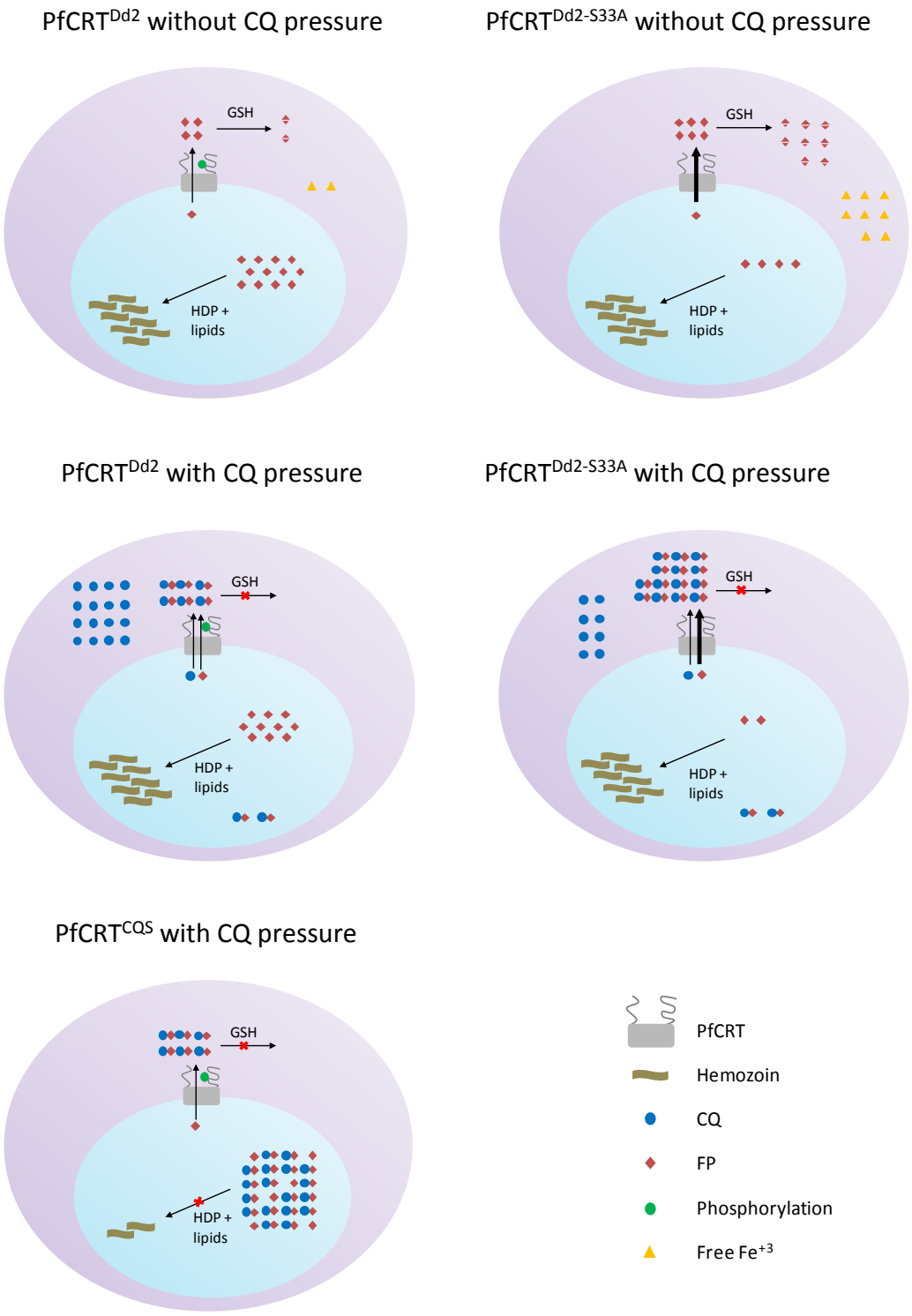


Figure 4.1. Transport of FP by PfCRT as a model to explain the PfCRT^{S33A} phenotype.

If the mutated PfCRT^{S33A} would transport FP more efficiently than the PfCRT haplotype of Dd2, this could increase the fitness of the parasite under normal culture conditions because a higher proportion of FP could be detoxified in the cytosol by glutathione and peroxide, and higher amounts or Fe^{+3} would be available for the parasite.

On the other hand, in the presence of CQ, the increased free FP concentration in the cytosol could be sequestered by CQ and, once bound to the drug, could not be detoxified.

This could explain why the mutant strain PFCRT^{S33A} is more sensitive to CQ than the parental Dd2. It has been shown that FP can be degraded *in vitro* by glutathione and that this detoxification process can be inhibited by CQ and amodiaquine (Ginsburg et al., 1998). Furthermore, higher levels of glutathione have been detected in CQ-resistant strains (Meierjohann et al., 2002) and it has also been shown that FP binds to and inhibits the cytosolic enzyme PfGAPDH (Campanale et al., 2003).

To investigate this hypothesis, the most straight-forward experiment to do would be uptake assays using radiolabelled FP in the *X. laevis* oocyte system because competitive experiments of CQ transport with FP would not be able to distinguish between CQ-FP dimer formation and competitive inhibition. Another key experiment would be to determine the IC_{50} of a panel of drugs including compounds targeting different parasite pathways. The PfCRT^{S33A} strain should be more sensitive only to drugs with an effect on heme detoxification. It would also be interesting to perform a fitness experiment in presence of QN as well as of another drug unrelated to heme detoxification, to confirm that the mutants are more susceptible only to drugs that target heme detoxification.

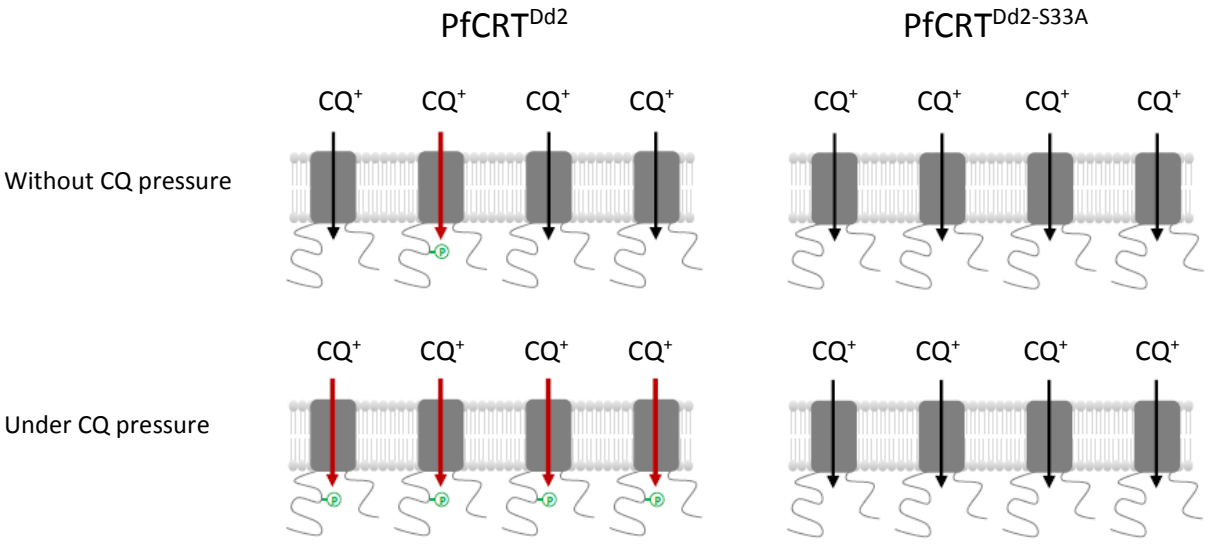


Figure 4.2. Upregulation of PfCRT phosphorylation under CQ pressure as a model to explain the PfCRT^{S33A} phenotype.

Another hypothesis is that CQ and QN accumulate more in the PfCRT^{S33A} strain than in the parental strain Dd2, but this difference is not detectable in a short term assay. The

transporter could be regulated by phosphorylation only when the parasite is under drug pressure. Phosphorylation is a dynamic process and it is regulated by the complementary activities of protein kinases and protein phosphatases. In this case, after a certain time of drug exposure, PfCRT would be phosphorylated and this would increase the rate of drug transport out of the food vacuole. Under drug pressure, the $PfCRT^{S33A}$ mutant could not be phosphorylated and hence, would accumulate more CQ than the parental strain Dd2. To test this hypothesis, uptake experiments for both strains previously cultured under drug pressure could be performed. Nevertheless, the outcome of this experiment is uncertain, because of the CQ trans-stimulation effect on PfCRT (Sanchez et al., 2003).

4.2.5. Relevance of the study

If the results of this study are confirmed by further experiments, it would be the first report of a *Plasmodium* transporter whose activity is regulated by a post-translational modification. This result would open the door to new intervention strategies towards the fight against malaria resistance. New kinase inhibitors could be identified in order to revert drug resistance or be used in combination therapies in order to avoid resistance development.

Already in 1987, verapamil was identified as CQ chemosensitizer (Martin et al., 1987) although its cardiac toxicity in humans prevented its clinical application. Since then, several compounds have proven to inhibit PfCRT-mediated CQ transport but none of them is currently under development (Martin et al., 2012; Ch'ng et al., 2013). Amlodipine, for example, exhibits poor pharmacokinetics properties that restrict its use in humans although it is effective in the animal model (Pereira et al., 2011). To overcome the poor potency of these compounds, the combination of several chemosensitizers has also been proposed as a strategy to restore CQ efficacy (van Schalkwyk et al., 2001). However, the reintroduction of CQ as antimalarial drug is questionable.

On the other hand, new aminoquinolines are undergoing preclinical trials and these new compounds could be administered in combination with PfCRT inhibitors. Potential kinase inhibitors could, in theory, display antimalarial activity and therefore kill the parasite and avoid PfCRT-mediated resistance at the same time, when used in combination with other drugs.

5. Outlook

The *X. laevis* oocyte system might not be an appropriate method to use for the characterization of the transport properties of PFE0825w. Nevertheless, there are alternative techniques that have been successfully used to express and study organic cation transporters that could be applied to this particular protein. The different techniques include expression in mammalian cell lines as BALB/3T3 (Sinclair et al., 2000) or HeLa (Zhang et al., 2000) and expression in yeast cells (Brosseau et al., 2015) or reconstituted proteoliposomes (Pochini et al., 2012).

Regarding the role of phosphorylation in PfCRT function, the generation of mutant lines carrying the $PfCRT^{S33D}$ and $PfCRT^{S33E}$ haplotypes should be a priority. Besides, the IC_{50} determination of a panel of drugs including compounds targeting different parasite pathways would support the fact that $PfCRT^{S33A}$ has an effect only on heme detoxification targeting drugs. On the same line, additional fitness experiments should be done in presence of QN and another unrelated antimalarial drug to confirm this result. Furthermore, to test the hypothesis that PfCRT may be regulated by phosphorylation under drug pressure, uptake experiments of the parental strain Dd2 and the mutant strain $PfCRT^{S33A}$ should be performed after longer exposure of the parasite lines to CQ and QN.

On the other hand, to investigate whether ML-7 has a direct effect on PfCRT or on PfMDR1, it would be advisable to study the phenotype of genetic engineered HB3 parasite lines carrying the $pfcrt^{Dd2}$or $pfmdr1^{Dd2}$genes in presence of ML-7.

Concerning PF11_0488, alternative strategies could be used to downregulate this protein, like the DD system. Instead, a conditional knock out could also be attempted using the diCre system to reject or confirm the interaction of this kinase with PfCRT. Finally and in order to identify new kinase candidates that could phosphorylate PfCRT, the recently developed technology called BioID that enables to study molecular interactions in its cellular environment could be applied for this transporter.

6. References

Abu Bakar, N., Klonis, N., Hanssen, E., Chan, C., and Tilley, L. (2010). Digestive-vacuole genesis and endocytic processes in the early intraerythrocytic stages of Plasmodium falciparum. Journal of cell science *123*, 441-450.

Adjalley, S.H., Lee, M.C., and Fidock, D.A. (2010). A method for rapid genetic integration into Plasmodium falciparum utilizing mycobacteriophage Bxb1 integrase. Methods in molecular biology *634*, 87-100.

Adjalley, S.H., Scanfeld, D., Kozlowski, E., Llinas, M., and Fidock, D.A. (2015). Genome-wide transcriptome profiling reveals functional networks involving the Plasmodium falciparum drug resistance transporters PfCRT and PfMDR1. BMC genomics *16*, 1090.

Aftab, D.T., Yang, J.M., and Hait, W.N. (1994). Functional role of phosphorylation of the multidrug transporter (P-glycoprotein) by protein kinase C in multidrug-resistant MCF-7 cells. Oncology research *6*, 59-70.

Amanchy, R., Periaswamy, B., Mathivanan, S., Reddy, R., Tattikota, S.G., and Pandey, A. (2007). A curated compendium of phosphorylation motifs. Nature biotechnology *25*, 285-286.

Amaratunga, C., Lim, P., Suon, S., Sreng, S., Mao, S., Sopha, C., Sam, B., Dek, D., Try, V., Amato, R.*, et al.* (2016). Dihydroartemisinin-piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study. The Lancet Infectious diseases *16*, 357-365.

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006). Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nature medicine *12*, 220-224.

Ancelin, M.L., Calas, M., Bompart, J., Cordina, G., Martin, D., Ben Bari, M., Jei, T., Druilhe, P., and Vial, H.J. (1998). Antimalarial activity of 77 phospholipid polar head analogs: close correlation between inhibition of phospholipid metabolism and in vitro Plasmodium falciparum growth. Blood *91*, 1426-1437.

Andriantsoanirina, V., Menard, D., Rabearimanana, S., Hubert, V., Bouchier, C., Tichit, M., Bras, J.L., and Durand, R. (2010). Association of microsatellite variations of Plasmodium falciparum Na+/H+ exchanger (Pfnhe-1) gene with reduced in vitro susceptibility to quinine: lack of confirmation in clinical isolates from Africa. The American journal of tropical medicine and hygiene *82*, 782-787.

Arastu-Kapur, S., Ponder, E.L., Fonovic, U.P., Yeoh, S., Yuan, F., Fonovic, M., Grainger, M., Phillips, C.I., Powers, J.C., and Bogyo, M. (2008). Identification of proteases that regulate erythrocyte rupture by the malaria parasite Plasmodium falciparum. Nature chemical biology *4*, 203-213.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L.*, et al.* (2014). A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature *505*, 50-55.

Arii, J., Goto, H., Suenaga, T., Oyama, M., Kozuka-Hata, H., Imai, T., Minowa, A., Akashi, H., Arase, H., Kawaoka, Y.*, et al.* (2010). Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. Nature *467*, 859-862.

Armstrong, C.M., and Goldberg, D.E. (2007). An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. Nature methods *4*, 1007-1009.

Avery, V.M., Bashyam, S., Burrows, J.N., Duffy, S., Papadatos, G., Puthukkuti, S., Sambandan, Y., Singh, S., Spangenberg, T., Waterson, D.*, et al.* (2014). Screening and hit evaluation of a chemical library against blood-stage Plasmodium falciparum. Malaria journal *13*, 190.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. The Biochemical journal *371*, 199-204.

Banerjee, R., Liu, J., Beatty, W., Pelosof, L., Klemba, M., and Goldberg, D.E. (2002). Four plasmepsins are active in the Plasmodium falciparum food vacuole, including a protease with an active-site histidine. Proceedings of the National Academy of Sciences of the United States of America *99*, 990-995.

Baum, J., Papenfuss, A.T., Mair, G.R., Janse, C.J., Vlachou, D., Waters, A.P., Cowman, A.F., Crabb, B.S., and de Koning-Ward, T.F. (2009). Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. Nucleic acids research *37*, 3788-3798.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T.W., Green, J.L., Holder, A.A., and Cowman, A.F. (2006). A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. The Journal of biological chemistry *281*, 5197-5208.

Beck, J.R., Muralidharan, V., Oksman, A., and Goldberg, D.E. (2014). PTEX component HSP101 mediates export of diverse malaria effectors into host erythrocytes. Nature *511*, 592-595.

Bellanca, S., Summers, R.L., Meyrath, M., Dave, A., Nash, M.N., Dittmer, M., Sanchez, C.P., Stein, W.D., Martin, R.E., and Lanzer, M. (2014). Multiple drugs compete for transport via the Plasmodium falciparum chloroquine resistance transporter at distinct but interdependent sites. The Journal of biological chemistry *289*, 36336-36351.

Ben Mamoun, C., Prigge, S.T., and Vial, H. (2010). Targeting the Lipid Metabolic Pathways for the Treatment of Malaria. Drug development research *71*, 44-55.

Bennett, T.N., Patel, J., Ferdig, M.T., and Roepe, P.D. (2007). Plasmodium falciparum Na+/H+ exchanger activity and quinine resistance. Molecular and biochemical parasitology *153*, 48-58.

Bhasin, V.K., and Trager, W. (1984). Gametocyte-forming and non-gametocyte-forming clones of Plasmodium falciparum. The American journal of tropical medicine and hygiene *33*, 534-537.

Biagini, G.A., Pasini, E.M., Hughes, R., De Koning, H.P., Vial, H.J., O'Neill, P.M., Ward, S.A., and Bray, P.G. (2004). Characterization of the choline carrier of Plasmodium falciparum: a route for the selective delivery of novel antimalarial drugs. Blood *104*, 3372-3377.

Biagini, G.A., Richier, E., Bray, P.G., Calas, M., Vial, H., and Ward, S.A. (2003). Heme binding contributes to antimalarial activity of bis-quaternary ammoniums. Antimicrobial agents and chemotherapy *47*, 2584-2589.

Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004). Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. Cell *117*, 503-514.

Birago, C., Albanesi, V., Silvestrini, F., Picci, L., Pizzi, E., Alano, P., Pace, T., and Ponzi, M. (2003). A gene-family encoding small exported proteins is conserved across Plasmodium genus. Molecular and biochemical parasitology *126*, 209-218.

Bolchoz, L.J., Budinsky, R.A., McMillan, D.C., and Jollow, D.J. (2001). Primaquine-induced hemolytic anemia: formation and hemotoxicity of the arylhydroxylamine metabolite 6-methoxy-8-hydroxylaminoquinoline. The Journal of pharmacology and experimental therapeutics *297*, 509-515.

Boncoeur, E., Durmort, C., Bernay, B., Ebel, C., Di Guilmi, A.M., Croize, J., Vernet, T., and Jault, J.M. (2012). PatA and PatB form a functional heterodimeric ABC multidrug efflux transporter responsible for the resistance of Streptococcus pneumoniae to fluoroquinolones. Biochemistry *51*, 7755-7765.

Bouyer, G., Reininger, L., Ramdani, G., L, D.P., Sharma, V., Egee, S., Langsley, G., and Lasonder, E. (2016). Plasmodiumfalciparum infection induces dynamic changes in the erythrocyte phospho-proteome. Blood cells, molecules & diseases *58*, 35-44.

Bray, P.G., Deed, S., Fox, E., Kalkanidis, M., Mungthin, M., Deady, L.W., and Tilley, L. (2005). Primaquine synergises the activity of chloroquine against chloroquine-resistant P. falciparum. Biochemical pharmacology *70*, 1158-1166.

Bray, P.G., Mungthin, M., Ridley, R.G., and Ward, S.A. (1998). Access to hematin: the basis of chloroquine resistance. Molecular pharmacology *54*, 170-179.

Brosseau, N., Andreev, E., and Ramotar, D. (2015). Complementation of the Yeast Model System Reveals that Caenorhabditis elegans OCT-1 Is a Functional Transporter of Anthracyclines. PloS one 10, e0133182.

Brown, S.B., Hatzikonstantinou, H., and Herries, D.G. (1978). The role of peroxide in haem degradation. A study of the oxidation of ferrihaems by hydrogen peroxide. The Biochemical journal *174*, 901-907.

Bruce, M.C., Alano, P., Duthie, S., and Carter, R. (1990). Commitment of the malaria parasite Plasmodium falciparum to sexual and asexual development. Parasitology *100 Pt 2*, 191-200.

Busch, A.E., Quester, S., Ulzheimer, J.C., Waldegger, S., Gorboulev, V., Arndt, P., Lang, F., and Koepsell, H. (1996). Electrogenic properties and substrate specificity of the polyspecific rat cation transporter rOCT1. The Journal of biological chemistry *271*, 32599-32604.

Campanale, N., Nickel, C., Daubenberger, C.A., Wehlan, D.A., Gorman, J.J., Klonis, N., Becker, K., and Tilley, L. (2003). Identification and characterization of heme-interacting proteins in the malaria parasite, Plasmodium falciparum. The Journal of biological chemistry *278*, 27354-27361.

Ch'ng, J.H., Mok, S., Bozdech, Z., Lear, M.J., Boudhar, A., Russell, B., Nosten, F., and Tan, K.S. (2013). A whole cell pathway screen reveals seven novel chemosensitizers to combat chloroquine resistant malaria. Scientific reports 3, 1734.

Cogswell, F.B., Collins, W.E., Krotoski, W.A., and Lowrie, R.C., Jr. (1991). Hypnozoites of Plasmodium simiovale. The American journal of tropical medicine and hygiene *45*, 211-213.

Collins, C.R., Das, S., Wong, E.H., Andenmatten, N., Stallmach, R., Hackett, F., Herman, J.P., Muller, S., Meissner, M., and Blackman, M.J. (2013a). Robust inducible Cre recombinase activity in the human malaria parasite Plasmodium falciparum enables efficient gene deletion within a single asexual erythrocytic growth cycle. Molecular microbiology *88*, 687-701.

Collins, C.R., Hackett, F., Strath, M., Penzo, M., Withers-Martinez, C., Baker, D.A., and Blackman, M.J. (2013b). Malaria parasite cGMP-dependent protein kinase regulates blood stage merozoite secretory organelle discharge and egress. PLoS pathogens *9*, e1003344.

Connell, L.E., and Helfman, D.M. (2006). Myosin light chain kinase plays a role in the regulation of epithelial cell survival. Journal of cell science *119*, 2269-2281.

Cooper, R.A., Lane, K.D., Deng, B., Mu, J., Patel, J.J., Wellems, T.E., Su, X., and Ferdig, M.T. (2007). Mutations in transmembrane domains 1, 4 and 9 of the Plasmodium falciparum chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. Molecular microbiology *63*, 270-282.

Coteron, J.M., Marco, M., Esquivias, J., Deng, X., White, K.L., White, J., Koltun, M., El Mazouni, F., Kokkonda, S., Katneni, K*., et al.* (2011). Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent Plasmodium falciparum dihydroorotate dehydrogenase inhibitors with clinical candidate potential. Journal of medicinal chemistry *54*, 5540-5561.

Cowman, A.F., Karcz, S., Galatis, D., and Culvenor, J.G. (1991). A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. The Journal of cell biology *113*, 1033-1042.

Crabb, B.S., and Cowman, A.F. (1996). Characterization of promoters and stable transfection by homologous and nonhomologous recombination in Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America *93*, 7289-7294.

Crabb, B.S., Rug, M., Gilberger, T.W., Thompson, J.K., Triglia, T., Maier, A.G., and Cowman, A.F. (2004). Transfection of the human malaria parasite Plasmodium falciparum. Methods in molecular biology *270*, 263-276.

Cuiv, P.O., Keogh, D., Clarke, P., and O'Connell, M. (2008). The hmuUV genes of Sinorhizobium meliloti 2011 encode the permease and ATPase components of an ABC transport system for the utilization of both haem and the hydroxamate siderophores, ferrichrome and ferrioxamine B. Molecular microbiology 70, 1261-1273.

Cyrklaff, M., Sanchez, C.P., Kilian, N., Bisseye, C., Simpore, J., Frischknecht, F., and Lanzer, M. (2011). Hemoglobins S and C interfere with actin remodeling in Plasmodium falciparum-infected erythrocytes. Science *334*, 1283-1286.

Chandramohanadas, R., Davis, P.H., Beiting, D.P., Harbut, M.B., Darling, C., Velmourougane, G., Lee, M.Y., Greer, P.A., Roos, D.S., and Greenbaum, D.C. (2009). Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. Science *324*, 794-797.

Chang, H.H., Falick, A.M., Carlton, P.M., Sedat, J.W., DeRisi, J.L., and Marletta, M.A. (2008). N-terminal processing of proteins exported by malaria parasites. Molecular and biochemical parasitology *160*, 107-115.

Chen, A.L., Kim, E.W., Toh, J.Y., Vashisht, A.A., Rashoff, A.Q., Van, C., Huang, A.S., Moon, A.S., Bell, H.N., Bentolila, L.A.*, et al.* (2015). Novel components of the Toxoplasma inner membrane complex revealed by BioID. mBio *6*, e02357-02314.

Chou, A.C., Chevli, R., and Fitch, C.D. (1980). Ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites. Biochemistry *19*, 1543-1549.

Chugh, M., Sundararaman, V., Kumar, S., Reddy, V.S., Siddiqui, W.A., Stuart, K.D., and Malhotra, P. (2013). Protein complex directs hemoglobin-to-hemozoin formation in Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America *110*, 5392-5397.

Dastidar, E.G., Dayer, G., Holland, Z.M., Dorin-Semblat, D., Claes, A., Chene, A., Sharma, A., Hamelin, R., Moniatte, M., Lopez-Rubio, J.J.*, et al.* (2012). Involvement of Plasmodium falciparum protein kinase CK2 in the chromatin assembly pathway. BMC biology *10*, 5.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. The Biochemical journal *351*, 95-105.

de Koning-Ward, T.F., Gilson, P.R., and Crabb, B.S. (2015). Advances in molecular genetic systems in malaria. Nature reviews Microbiology *13*, 373-387.

Doherty, J.P., Lindeman, R., Trent, R.J., Graham, M.W., and Woodcock, D.M. (1993). Escherichia coli host strains SURE and SRB fail to preserve a palindrome cloned in lambda phage: improved alternate host strains. Gene *124*, 29-35.

Dubar, F., Egan, T.J., Pradines, B., Kuter, D., Ncokazi, K.K., Forge, D., Paul, J.F., Pierrot, C., Kalamou, H., Khalife, J.*, et al.* (2011). The antimalarial ferroquine: role of the metal and intramolecular hydrogen bond in activity and resistance. ACS chemical biology *6*, 275-287.

Dumont, J.N. (1972). Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals. Journal of morphology *136*, 153-179.

Duncan, J.S., Gyenis, L., Lenehan, J., Bretner, M., Graves, L.M., Haystead, T.A., and Litchfield, D.W. (2008). An unbiased evaluation of CK2 inhibitors by chemoproteomics: characterization of inhibitor effects on CK2 and identification of novel inhibitor targets. Molecular & cellular proteomics : MCP *7*, 1077-1088.

Duraisingh, M.T., Drakeley, C.J., Muller, O., Bailey, R., Snounou, G., Targett, G.A., Greenwood, B.M., and Warhurst, D.C. (1997). Evidence for selection for the tyrosine-86 allele of the pfmdr 1 gene of Plasmodium falciparum by chloroquine and amodiaquine. Parasitology *114 (Pt 3)*, 205-211.

Duraisingh, M.T., Triglia, T., and Cowman, A.F. (2002). Negative selection of Plasmodium falciparum reveals targeted gene deletion by double crossover recombination. International journal for parasitology *32*, 81-89.

Eckstein-Ludwig, U., Webb, R.J., Van Goethem, I.D., East, J.M., Lee, A.G., Kimura, M., O'Neill, P.M., Bray, P.G., Ward, S.A., and Krishna, S. (2003). Artemisinins target the SERCA of Plasmodium falciparum. Nature *424*, 957-961.

Echeverry, D.F., Holmgren, G., Murillo, C., Higuita, J.C., Bjorkman, A., Gil, J.P., and Osorio, L. (2007). Short report: polymorphisms in the pfcrt and pfmdr1 genes of Plasmodium falciparum and in vitro susceptibility to amodiaquine and desethylamodiaquine. The American journal of tropical medicine and hygiene *77*, 1034-1038.

Egan, T.J., Combrinck, J.M., Egan, J., Hearne, G.R., Marques, H.M., Ntenteni, S., Sewell, B.T., Smith, P.J., Taylor, D., van Schalkwyk, D.A*., et al.* (2002). Fate of haem iron in the malaria parasite Plasmodium falciparum. The Biochemical journal *365*, 343-347.

Egee, S., Lapaix, F., Decherf, G., Staines, H.M., Ellory, J.C., Doerig, C., and Thomas, S.L. (2002). A stretch-activated anion channel is up-regulated by the malaria parasite Plasmodium falciparum. The Journal of physiology *542*, 795-801.

Eggleson, K.K., Duffin, K.L., and Goldberg, D.E. (1999). Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite Plasmodium falciparum. The Journal of biological chemistry *274*, 32411-32417.

El Bissati, K., Downie, M.J., Kim, S.K., Horowitz, M., Carter, N., Ullman, B., and Ben Mamoun, C. (2008). Genetic evidence for the essential role of PfNT1 in the transport and utilization of xanthine, guanine, guanosine and adenine by Plasmodium falciparum. Molecular and biochemical parasitology *161*, 130-139.

Elsworth, B., Matthews, K., Nie, C.Q., Kalanon, M., Charnaud, S.C., Sanders, P.R., Chisholm, S.A., Counihan, N.A., Shaw, P.J., Pino, P*., et al.* (2014). PTEX is an essential nexus for protein export in malaria parasites. Nature *511*, 587-591.

Fennell, C., Babbitt, S., Russo, I., Wilkes, J., Ranford-Cartwright, L., Goldberg, D.E., and Doerig, C. (2009). PfeIK1, a eukaryotic initiation factor 2alpha kinase of the human malaria parasite Plasmodium falciparum, regulates stress-response to amino-acid starvation. Malaria journal *8*, 99.

Ferdig, M.T., Cooper, R.A., Mu, J., Deng, B., Joy, D.A., Su, X.Z., and Wellems, T.E. (2004). Dissecting the loci of low-level quinine resistance in malaria parasites. Molecular microbiology *52*, 985-997.

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naude, B., Deitsch, K.W*., et al.* (2000). Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Molecular cell *6*, 861-871.

Fitch, C.D. (1986). Antimalarial schizontocides: ferriprotoporphyrin IX interaction hypothesis. Parasitology today *2*, 330-331.

Fitch, C.D., and Chou, A.C. (1997). Regulation of heme polymerizing activity and the antimalarial action of chloroquine. Antimicrobial agents and chemotherapy *41*, 2461-2465.

Freitas-Junior, L.H., Hernandez-Rivas, R., Ralph, S.A., Montiel-Condado, D., Ruvalcaba-Salazar, O.K., Rojas-Meza, A.P., Mancio-Silva, L., Leal-Silvestre, R.J., Gontijo, A.M., Shorte, S.*, et al.* (2005). Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. Cell *121*, 25-36.

Frevert, U., Engelmann, S., Zougbede, S., Stange, J., Ng, B., Matuschewski, K., Liebes, L., and Yee, H. (2005). Intravital observation of Plasmodium berghei sporozoite infection of the liver. PLoS biology 3, e192.

Frevert, U., Sinnis, P., Cerami, C., Shreffler, W., Takacs, B., and Nussenzweig, V. (1993). Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. The Journal of experimental medicine *177*, 1287-1298.

Fry, M., and Pudney, M. (1992). Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). Biochemical pharmacology *43*, 1545-1553.

Gamo, F.J., Sanz, L.M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J.L., Vanderwall, D.E., Green, D.V., Kumar, V., Hasan, S.*, et al.* (2010). Thousands of chemical starting points for antimalarial lead identification. Nature *465*, 305-310.

Gavigan, C.S., Dalton, J.P., and Bell, A. (2001). The role of aminopeptidases in haemoglobin degradation in Plasmodium falciparum-infected erythrocytes. Molecular and biochemical parasitology *117*, 37-48.

Ghorbal, M., Gorman, M., Macpherson, C.R., Martins, R.M., Scherf, A., and Lopez-Rubio, J.J. (2014). Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. Nature biotechnology *32*, 819-821.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A.*, et al.* (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell *154*, 442-451.

Ginsburg, H., Famin, O., Zhang, J., and Krugliak, M. (1998). Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. Biochemical pharmacology *56*, 1305-1313.

Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L., and Goldberg, D.E. (1994). Order and specificity of the Plasmodium falciparum hemoglobin degradation pathway. The Journal of clinical investigation *93*, 1602-1608.

Goel, V.K., Li, X., Chen, H., Liu, S.C., Chishti, A.H., and Oh, S.S. (2003). Band 3 is a host receptor binding merozoite surface protein 1 during the Plasmodium falciparum invasion of erythrocytes. Proceedings of the National Academy of Sciences of the United States of America *100*, 5164-5169.

Goldfless, S.J., Wagner, J.C., and Niles, J.C. (2014). Versatile control of Plasmodium falciparum gene expression with an inducible protein-RNA interaction. Nature communications *5*, 5329.

Goldin, A.L. (1992). Maintenance of Xenopus laevis and oocyte injection. Methods in enzymology 207, 266-279.

Graciotti, M., Alam, M., Solyakov, L., Schmid, R., Burley, G., Bottrill, A.R., Doerig, C., Cullis, P., and Tobin, A.B. (2014). Malaria protein kinase CK2 (PfCK2) shows novel mechanisms of regulation. PloS one *9*, e85391.

Graf, G.A., Cohen, J.C., and Hobbs, H.H. (2004). Missense mutations in ABCG5 and ABCG8 disrupt heterodimerization and trafficking. The Journal of biological chemistry *279*, 24881-24888.

Gregson, A., and Plowe, C.V. (2005). Mechanisms of resistance of malaria parasites to antifolates. Pharmacological reviews *57*, 117-145.

Grundemann, D., Gorboulev, V., Gambaryan, S., Veyhl, M., and Koepsell, H. (1994). Drug excretion mediated by a new prototype of polyspecific transporter. Nature *372*, 549-552.

Gruring, C., Heiber, A., Kruse, F., Ungefehr, J., Gilberger, T.W., and Spielmann, T. (2011). Development and host cell modifications of Plasmodium falciparum blood stages in four dimensions. Nature communications *2*, 165.

Guinet, F., Dvorak, J.A., Fujioka, H., Keister, D.B., Muratova, O., Kaslow, D.C., Aikawa, M., Vaidya, A.B., and Wellems, T.E. (1996). A developmental defect in Plasmodium falciparum male gametogenesis. The Journal of cell biology *135*, 269-278.

Gurdon, J.B., Lane, C.D., Woodland, H.R., and Marbaix, G. (1971). Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. Nature *233*, 177-182.

Hadley, T.J., Klotz, F.W., Pasvol, G., Haynes, J.D., McGinniss, M.H., Okubo, Y., and Miller, L.H. (1987). Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. The Journal of clinical investigation *80*, 1190-1193.

Hansen, M., Kun, J.F., Schultz, J.E., and Beitz, E. (2002). A single, bi-functional aquaglyceroporin in blood-stage Plasmodium falciparum malaria parasites. The Journal of biological chemistry *277*, 4874-4882.

Hanspal, M., Dua, M., Takakuwa, Y., Chishti, A.H., and Mizuno, A. (2002). Plasmodium falciparum cysteine protease falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. Blood *100*, 1048-1054.

Hawley, S.R., Bray, P.G., Mungthin, M., Atkinson, J.D., O'Neill, P.M., and Ward, S.A. (1998). Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in Plasmodium falciparum in vitro. Antimicrobial agents and chemotherapy *42*, 682-686.

Hilden, I., Leggio, L.L., Larsen, S., and Poulsen, P. (2000). Characterization and crystallization of an active N-terminally truncated form of the Escherichia coli glycogen branching enzyme. European journal of biochemistry / FEBS 267, 2150-2155.

Holland, Z., Prudent, R., Reiser, J.B., Cochet, C., and Doerig, C. (2009). Functional analysis of protein kinase CK2 of the human malaria parasite Plasmodium falciparum. Eukaryotic cell *8*, 388-397.

Hoppe, H.C., van Schalkwyk, D.A., Wiehart, U.I., Meredith, S.A., Egan, J., and Weber, B.W. (2004). Antimalarial quinolines and artemisinin inhibit endocytosis in Plasmodium falciparum. Antimicrobial agents and chemotherapy *48*, 2370-2378.

Howe, R., Kelly, M., Jimah, J., Hodge, D., and Odom, A.R. (2013). Isoprenoid biosynthesis inhibition disrupts Rab5 localization and food vacuolar integrity in Plasmodium falciparum. Eukaryotic cell *12*, 215-223.

Huber, S.M., Uhlemann, A.C., Gamper, N.L., Duranton, C., Kremsner, P.G., and Lang, F. (2002). Plasmodium falciparum activates endogenous Cl(-) channels of human erythrocytes by membrane oxidation. The EMBO journal *21*, 22-30.

Imwong, M., Snounou, G., Pukrittayakamee, S., Tanomsing, N., Kim, J.R., Nandy, A., Guthmann, J.P., Nosten, F., Carlton, J., Looareesuwan, S*., et al.* (2007). Relapses of Plasmodium vivax infection usually result from activation of heterologous hypnozoites. The Journal of infectious diseases *195*, 927-933.

Ishino, T., Chinzei, Y., and Yuda, M. (2005). Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. Molecular microbiology *58*, 1264-1275.

Issar, N., Roux, E., Mattei, D., and Scherf, A. (2008). Identification of a novel post-translational modification in Plasmodium falciparum: protein sumoylation in different cellular compartments. Cellular microbiology *10*, 1999-2011.

Ito, S., Richert, N., and Pastan, I. (1982). Vinculin phosphorylation by the src kinase: inhibition by chlorpromazine, imipramine and local anesthetics. Biochemical and biophysical research communications *107*, 670-675.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proceedings of the National Academy of Sciences of the United States of America *98*, 4569-4574.

Jani, D., Nagarkatti, R., Beatty, W., Angel, R., Slebodnick, C., Andersen, J., Kumar, S., and Rathore, D. (2008). HDP-a novel heme detoxification protein from the malaria parasite. PLoS pathogens *4*, e1000053.

Jimenez-Diaz, M.B., Ebert, D., Salinas, Y., Pradhan, A., Lehane, A.M., Myrand-Lapierre, M.E., O'Loughlin, K.G., Shackleford, D.M., Justino de Almeida, M., Carrillo, A.K*., et al.* (2014). (+)-SJ733, a clinical candidate for malaria that acts through ATP4 to induce rapid host-mediated clearance of Plasmodium. Proceedings of the National Academy of Sciences of the United States of America *111*, E5455-5462.

Jimenez, E., Nunez, E., Ibanez, I., Draffin, J.E., Zafra, F., and Gimenez, C. (2014). Differential regulation of the glutamate transporters GLT-1 and GLAST by GSK3beta. Neurochemistry international *79*, 33-43.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*, 816-821.

Johnson, D.J., Fidock, D.A., Mungthin, M., Lakshmanan, V., Sidhu, A.B., Bray, P.G., and Ward, S.A. (2004). Evidence for a central role for PfCRT in conferring Plasmodium falciparum resistance to diverse antimalarial agents. Molecular cell *15*, 867-877.

Joice, R., Nilsson, S.K., Montgomery, J., Dankwa, S., Egan, E., Morahan, B., Seydel, K.B., Bertuccini, L., Alano, P., Williamson, K.C*., et al.* (2014). Plasmodium falciparum transmission stages accumulate in the human bone marrow. Science translational medicine *6*, 244re245.

Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H.K*., et al.* (1999). Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science *285*, 1573-1576.

Jones, M.L., Collins, M.O., Goulding, D., Choudhary, J.S., and Rayner, J.C. (2012). Analysis of protein palmitoylation reveals a pervasive role in Plasmodium development and pathogenesis. Cell host & microbe *12*, 246-258.

Josling, G.A., and Llinas, M. (2015). Sexual development in Plasmodium parasites: knowing when it's time to commit. Nature reviews Microbiology *13*, 573-587.

Joucla, G., Pizzut, S., Monsan, P., and Remaud-Simeon, M. (2006). Construction of a fully active truncated alternansucrase partially deleted of its carboxy-terminal domain. FEBS letters 580, 763-768.

Juge, N., Moriyama, S., Miyaji, T., Kawakami, M., Iwai, H., Fukui, T., Nelson, N., Omote, H., and Moriyama, Y. (2015). Plasmodium falciparum chloroquine resistance transporter is a H+-coupled polyspecific nutrient and drug exporter. Proceedings of the National Academy of Sciences of the United States of America 112, 3356-3361.

Kafsack, B.F., Rovira-Graells, N., Clark, T.G., Bancells, C., Crowley, V.M., Campino, S.G., Williams, A.E., Drought, L.G., Kwiatkowski, D.P., Baker, D.A*., et al.* (2014). A transcriptional switch underlies commitment to sexual development in malaria parasites. Nature *507*, 248-252.

Kakehi, M., Koyabu, N., Nakamura, T., Uchiumi, T., Kuwano, M., Ohtani, H., and Sawada, Y. (2002). Functional characterization of mouse cation transporter mOCT2 compared with mOCT1. Biochemical and biophysical research communications *296*, 644-650.

Karcz, S.R., Galatis, D., and Cowman, A.F. (1993). Nucleotide binding properties of a P-glycoprotein homologue from Plasmodium falciparum. Molecular and biochemical parasitology *58*, 269-276.

Kehr, S., Jortzik, E., Delahunty, C., Yates, J.R., 3rd, Rahlfs, S., and Becker, K. (2011). Protein S-glutathionylation in malaria parasites. Antioxidants & redox signaling *15*, 2855-2865.

Kimura, E.A., Couto, A.S., Peres, V.J., Casal, O.L., and Katzin, A.M. (1996). N-linked glycoproteins are related to schizogony of the intraerythrocytic stage in Plasmodium falciparum. The Journal of biological chemistry *271*, 14452-14461.

Kirk, K., Wong, H.Y., Elford, B.C., Newbold, C.I., and Ellory, J.C. (1991). Enhanced choline and Rb+ transport in human erythrocytes infected with the malaria parasite Plasmodium falciparum. The Biochemical journal *278 (Pt 2)*, 521-525.

Klokouzas, A., Tiffert, T., van Schalkwyk, D., Wu, C.P., van Veen, H.W., Barrand, M.A., and Hladky, S.B. (2004). Plasmodium falciparum expresses a multidrug resistance-associated protein. Biochemical and biophysical research communications *321*, 197-201.

Klonis, N., Creek, D.J., and Tilley, L. (2013). Iron and heme metabolism in Plasmodium falciparum and the mechanism of action of artemisinins. Current opinion in microbiology *16*, 722-727.

Krishna, S., Pulcini, S., Moore, C.M., Teo, B.H., and Staines, H.M. (2014). Pumped up: reflections on PfATP6 as the target for artemisinins. Trends in pharmacological sciences *35*, 4-11.

Krishna, S., Woodrow, C., Webb, R., Penny, J., Takeyasu, K., Kimura, M., and East, J.M. (2001). Expression and functional characterization of a Plasmodium falciparum Ca2+-ATPase (PfATP4) belonging to a subclass unique to apicomplexan organisms. The Journal of biological chemistry *276*, 10782-10787.

Krotoski, W.A., Garnham, P.C., Cogswell, F.B., Collins, W.E., Bray, R.S., Gwasz, R.W., Killick-Kendrick, R., Wolf, R.H., Sinden, R., Hollingdale, M*., et al.* (1986). Observations on early and late post-sporozoite tissue stages in primate malaria. IV. Pre-erythrocytic schizonts and/or hypnozoites of Chesson and North Korean strains of Plasmodium vivax in the chimpanzee. The American journal of tropical medicine and hygiene *35*, 263-274.

Krugliak, M., and Ginsburg, H. (2006). The evolution of the new permeability pathways in Plasmodium falciparum--infected erythrocytes--a kinetic analysis. Experimental parasitology *114*, 253-258.

Krugliak, M., Zhang, J., and Ginsburg, H. (2002). Intraerythrocytic Plasmodium falciparum utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. Molecular and biochemical parasitology *119*, 249-256.

Kublin, J.G., Cortese, J.F., Njunju, E.M., Mukadam, R.A., Wirima, J.J., Kazembe, P.N., Djimde, A.A., Kouriba, B., Taylor, T.E., and Plowe, C.V. (2003). Reemergence of chloroquine-sensitive Plasmodium falciparum malaria after cessation of chloroquine use in Malawi. The Journal of infectious diseases *187*, 1870-1875.

Kuhn, Y., Sanchez, C.P., Ayoub, D., Saridaki, T., van Dorsselaer, A., and Lanzer, M. (2010). Trafficking of the phosphoprotein PfCRT to the digestive vacuolar membrane in Plasmodium falciparum. Traffic *11*, 236-249.

Labaied, M., Camargo, N., and Kappe, S.H. (2007). Depletion of the Plasmodium berghei thrombospondin-related sporozoite protein reveals a role in host cell entry by sporozoites. Molecular and biochemical parasitology *153*, 158-166.

Lakshmanan, V., Bray, P.G., Verdier-Pinard, D., Johnson, D.J., Horrocks, P., Muhle, R.A., Alakpa, G.E., Hughes, R.H., Ward, S.A., Krogstad, D.J*., et al.* (2005). A critical role for PfCRT K76T in Plasmodium falciparum verapamil-reversible chloroquine resistance. The EMBO journal *24*, 2294-2305.

Lambros, C., and Vanderberg, J.P. (1979). Synchronization of Plasmodium falciparum erythrocytic stages in culture. The Journal of parasitology 65, 418-420.

Larkins, B.A., Pedersen, K., Handa, A.K., Hurkman, W.J., and Smith, L.D. (1979). Synthesis and processing of maize storage proteins in Xenopus laevis oocytes. Proceedings of the National Academy of Sciences of the United States of America *76*, 6448-6452.

Lasonder, E., Green, J.L., Camarda, G., Talabani, H., Holder, A.A., Langsley, G., and Alano, P. (2012). The Plasmodium falciparum schizont phosphoproteome reveals extensive phosphatidylinositol and cAMP-protein kinase A signaling. Journal of proteome research *11*, 5323-5337.

Lawrence, J.C., Jr., Hiken, J.F., and James, D.E. (1990). Stimulation of glucose transport and glucose transporter phosphorylation by okadaic acid in rat adipocytes. The Journal of biological chemistry *265*, 19768-19776.

Le Bonniec, S., Deregnaucourt, C., Redeker, V., Banerjee, R., Grellier, P., Goldberg, D.E., and Schrevel, J. (1999). Plasmepsin II, an acidic hemoglobinase from the Plasmodium falciparum food vacuole, is active at neutral pH on the host erythrocyte membrane skeleton. The Journal of biological chemistry *274*, 14218-14223.

Lee, A.H., Symington, L.S., and Fidock, D.A. (2014). DNA repair mechanisms and their biological roles in the malaria parasite Plasmodium falciparum. Microbiology and molecular biology reviews : MMBR *78*, 469-486.

Lehane, A.M., Saliba, K.J., Allen, R.J., and Kirk, K. (2004). Choline uptake into the malaria parasite is energized by the membrane potential. Biochemical and biophysical research communications *320*, 311-317.

Leykauf, K., Treeck, M., Gilson, P.R., Nebl, T., Braulke, T., Cowman, A.F., Gilberger, T.W., and Crabb, B.S. (2010). Protein kinase a dependent phosphorylation of apical membrane antigen 1 plays an important role in erythrocyte invasion by the malaria parasite. PLoS pathogens *6*, e1000941.

Lin, H.B., Cadete, V.J., Sawicka, J., Wozniak, M., and Sawicki, G. (2012). Effect of the myosin light chain kinase inhibitor ML-7 on the proteome of hearts subjected to ischemia-reperfusion injury. Journal of proteomics *75*, 5386-5395.

Liu, J., Istvan, E.S., Gluzman, I.Y., Gross, J., and Goldberg, D.E. (2006). Plasmodium falciparum ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proceedings of the National Academy of Sciences of the United States of America *103*, 8840-8845.

Lopez-Rubio, J.J., Gontijo, A.M., Nunes, M.C., Issar, N., Hernandez Rivas, R., and Scherf, A. (2007). 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. Molecular microbiology *66*, 1296-1305.

Lopez-Rubio, J.J., Mancio-Silva, L., and Scherf, A. (2009). Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. Cell host & microbe *5*, 179-190.

Loria, P., Miller, S., Foley, M., and Tilley, L. (1999). Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. The Biochemical journal *339 (Pt 2)*, 363-370.

Lu, F., Jiang, H., Ding, J., Mu, J., Valenzuela, J.G., Ribeiro, J.M., and Su, X.Z. (2007). cDNA sequences reveal considerable gene prediction inaccuracy in the Plasmodium falciparum genome. BMC genomics *8*, 255.

Lykke-Andersen, S., and Jensen, T.H. (2015). Nonsense-mediated mRNA decay: an intricate machinery that shapes transcriptomes. Nature reviews Molecular cell biology 16, 665-677.

Maier, A.G., Braks, J.A., Waters, A.P., and Cowman, A.F. (2006). Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination. Molecular and biochemical parasitology *150*, 118-121.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823-826.

Marchetti, R.V., Lehane, A.M., Shafik, S.H., Winterberg, M., Martin, R.E., and Kirk, K. (2015). A lactate and formate transporter in the intraerythrocytic malaria parasite, Plasmodium falciparum. Nature communications *6*, 6721.

Marti, M., Good, R.T., Rug, M., Knuepfer, E., and Cowman, A.F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science *306*, 1930-1933.

Martin, R.E., Butterworth, A.S., Gardiner, D.L., Kirk, K., McCarthy, J.S., and Skinner-Adams, T.S. (2012). Saquinavir inhibits the malaria parasite's chloroquine resistance transporter. Antimicrobial agents and chemotherapy 56, 2283-2289.

Martin, R.E., Henry, R.I., Abbey, J.L., Clements, J.D., and Kirk, K. (2005). The 'permeome' of the malaria parasite: an overview of the membrane transport proteins of Plasmodium falciparum. Genome biology *6*, R26.

Martin, R.E., and Kirk, K. (2004). The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. Molecular biology and evolution *21*, 1938-1949.

Martin, R.E., Marchetti, R.V., Cowan, A.I., Howitt, S.M., Broer, S., and Kirk, K. (2009). Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science *325*, 1680-1682.

Martin, S.K., Oduola, A.M., and Milhous, W.K. (1987). Reversal of chloroquine resistance in Plasmodium falciparum by verapamil. Science 235, 899-901.

Maughan, S.C., Pasternak, M., Cairns, N., Kiddle, G., Brach, T., Jarvis, R., Haas, F., Nieuwland, J., Lim, B., Muller, C.*, et al.* (2010). Plant homologs of the Plasmodium falciparum chloroquine-resistance transporter, PfCRT, are required for glutathione homeostasis and stress responses. Proceedings of the National Academy of Sciences of the United States of America *107*, 2331-2336.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R.V., Rizk, S.S., Njimoh, D.L., Ryan, Y., Chotivanich, K.*, et al.* (2015). A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. Nature *520*, 683-687.

McNamara, C.W., Lee, M.C., Lim, C.S., Lim, S.H., Roland, J., Nagle, A., Simon, O., Yeung, B.K., Chatterjee, A.K., McCormack, S.L.*, et al.* (2013). Targeting Plasmodium PI(4)K to eliminate malaria. Nature *504*, 248-253.

Mehlotra, R.K., Mattera, G., Bockarie, M.J., Maguire, J.D., Baird, J.K., Sharma, Y.D., Alifrangis, M., Dorsey, G., Rosenthal, P.J., Fryauff, D.J.*, et al.* (2008). Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite Plasmodium falciparum. Antimicrobial agents and chemotherapy *52*, 2212-2222.

Mehrens, T., Lelleck, S., Cetinkaya, I., Knollmann, M., Hohage, H., Gorboulev, V., Boknik, P., Koepsell, H., and Schlatter, E. (2000). The affinity of the organic cation transporter rOCT1 is increased

by protein kinase C-dependent phosphorylation. Journal of the American Society of Nephrology : JASN *11*, 1216-1224.

Meierjohann, S., Walter, R.D., and Muller, S. (2002). Regulation of intracellular glutathione levels in erythrocytes infected with chloroquine-sensitive and chloroquine-resistant Plasmodium falciparum. The Biochemical journal *368*, 761-768.

Meissner, M., Krejany, E., Gilson, P.R., de Koning-Ward, T.F., Soldati, D., and Crabb, B.S. (2005). Tetracycline analogue-regulated transgene expression in Plasmodium falciparum blood stages using Toxoplasma gondii transactivators. Proceedings of the National Academy of Sciences of the United States of America *102*, 2980-2985.

Meister, S., Plouffe, D.M., Kuhen, K.L., Bonamy, G.M., Wu, T., Barnes, S.W., Bopp, S.E., Borboa, R., Bright, A.T., Che, J.*, et al.* (2011). Imaging of Plasmodium liver stages to drive next-generation antimalarial drug discovery. Science *334*, 1372-1377.

Mi, L., Gan, N., Cheema, A., Dakshanamurthy, S., Wang, X., Yang, D.C., and Chung, F.L. (2009). Cancer preventive isothiocyanates induce selective degradation of cellular alpha- and beta-tubulins by proteasomes. The Journal of biological chemistry *284*, 17039-17051.

Mita, T., Kaneko, A., Lum, J.K., Zungu, I.L., Tsukahara, T., Eto, H., Kobayakawa, T., Bjorkman, A., and Tanabe, K. (2004). Expansion of wild type allele rather than back mutation in pfcrt explains the recent recovery of chloroquine sensitivity of Plasmodium falciparum in Malawi. Molecular and biochemical parasitology *135*, 159-163.

Moeller, H.B., Olesen, E.T., and Fenton, R.A. (2011). Regulation of the water channel aquaporin-2 by posttranslational modification. American journal of physiology Renal physiology *300*, F1062-1073.

Mok, S., Liong, K.Y., Lim, E.H., Huang, X., Zhu, L., Preiser, P.R., and Bozdech, Z. (2014). Structural polymorphism in the promoter of pfmrp2 confers Plasmodium falciparum tolerance to quinoline drugs. Molecular microbiology *91*, 918-934.

Moraes Barros, R.R., Straimer, J., Sa, J.M., Salzman, R.E., Melendez-Muniz, V.A., Mu, J., Fidock, D.A., and Wellems, T.E. (2015). Editing the Plasmodium vivax genome, using zinc-finger nucleases. The Journal of infectious diseases *211*, 125-129.

Moritz, A.E., Rastedt, D.E., Stanislowski, D.J., Shetty, M., Smith, M.A., Vaughan, R.A., and Foster, J.D. (2015). Reciprocal Phosphorylation and Palmitoylation Control Dopamine Transporter Kinetics. The Journal of biological chemistry *290*, 29095-29105.

Mu, J., Ferdig, M.T., Feng, X., Joy, D.A., Duan, J., Furuya, T., Subramanian, G., Aravind, L., Cooper, R.A., Wootton, J.C.*, et al.* (2003). Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Molecular microbiology *49*, 977-989.

Muangnoicharoen, S., Johnson, D.J., Looareesuwan, S., Krudsood, S., and Ward, S.A. (2009). Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin in vitro. Antimicrobial agents and chemotherapy *53*, 1362-1366.

Mullin, K.A., Lim, L., Ralph, S.A., Spurck, T.P., Handman, E., and McFadden, G.I. (2006). Membrane transporters in the relict plastid of malaria parasites. Proceedings of the National Academy of Sciences of the United States of America *103*, 9572-9577.

Muralidharan, V., Oksman, A., Iwamoto, M., Wandless, T.J., and Goldberg, D.E. (2011). Asparagine repeat function in a Plasmodium falciparum protein assessed via a regulatable fluorescent affinity tag. Proceedings of the National Academy of Sciences of the United States of America *108*, 4411-4416.

Mzayek, F., Deng, H., Mather, F.J., Wasilevich, E.C., Liu, H., Hadi, C.M., Chansolme, D.H., Murphy, H.A., Melek, B.H., Tenaglia, A.N.*, et al.* (2007). Randomized dose-ranging controlled trial of

AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. PLoS clinical trials *2*, e6.

Nagelschmitz, J., Voith, B., Wensing, G., Roemer, A., Fugmann, B., Haynes, R.K., Kotecka, B.M., Rieckmann, K.H., and Edstein, M.D. (2008). First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. Antimicrobial agents and chemotherapy *52*, 3085-3091.

Nasir ud, D., Drager-Dayal, R., Decrind, C., Hu, B.H., Del Giudice, G., and Hoessli, D. (1992). Plasmodium falciparum synthesizes O-glycosylated glycoproteins containing O-linked N-acetylglucosamine. Biochemistry international *27*, 55-64.

Naude, B., Brzostowski, J.A., Kimmel, A.R., and Wellems, T.E. (2005). Dictyostelium discoideum expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, Plasmodium falciparum transporter PfCRT. The Journal of biological chemistry *280*, 25596-25603.

Nestler, E.J., Terwilliger, R.Z., and Duman, R.S. (1989). Chronic antidepressant administration alters the subcellular distribution of cyclic AMP-dependent protein kinase in rat frontal cortex. Journal of neurochemistry *53*, 1644-1647.

Newby, Z.E., O'Connell, J., 3rd, Robles-Colmenares, Y., Khademi, S., Miercke, L.J., and Stroud, R.M. (2008). Crystal structure of the aquaglyceroporin PfAQP from the malarial parasite Plasmodium falciparum. Nature structural & molecular biology *15*, 619-625.

Nguitragool, W., Bokhari, A.A., Pillai, A.D., Rayavara, K., Sharma, P., Turpin, B., Aravind, L., and Desai, S.A. (2011). Malaria parasite clag3 genes determine channel-mediated nutrient uptake by infected red blood cells. Cell *145*, 665-677.

Niang, M., Yan Yam, X., and Preiser, P.R. (2009). The Plasmodium falciparum STEVOR multigene family mediates antigenic variation of the infected erythrocyte. PLoS pathogens *5*, e1000307.

Nkrumah, L.J., Muhle, R.A., Moura, P.A., Ghosh, P., Hatfull, G.F., Jacobs, W.R., Jr., and Fidock, D.A. (2006). Efficient site-specific integration in Plasmodium falciparum chromosomes mediated by mycobacteriophage Bxb1 integrase. Nature methods *3*, 615-621.

Nkrumah, L.J., Riegelhaupt, P.M., Moura, P., Johnson, D.J., Patel, J., Hayton, K., Ferdig, M.T., Wellems, T.E., Akabas, M.H., and Fidock, D.A. (2009). Probing the multifactorial basis of Plasmodium falciparum quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. Molecular and biochemical parasitology *165*, 122-131.

O'Neill, M.T., Phuong, T., Healer, J., Richard, D., and Cowman, A.F. (2011). Gene deletion from Plasmodium falciparum using FLP and Cre recombinases: implications for applied site-specific recombination. International journal for parasitology *41*, 117-123.

O'Neill, P.M., Amewu, R.K., Nixon, G.L., Bousejra ElGarah, F., Mungthin, M., Chadwick, J., Shone, A.E., Vivas, L., Lander, H., Barton, V.*, et al.* (2010a). Identification of a 1,2,4,5-tetraoxane antimalarial drug-development candidate (RKA 182) with superior properties to the semisynthetic artemisinins. Angewandte Chemie *49*, 5693-5697.

O'Neill, P.M., Barton, V.E., and Ward, S.A. (2010b). The molecular mechanism of action of artemisinin--the debate continues. Molecules *15*, 1705-1721.

Okuda, T., and Haga, T. (2000). Functional characterization of the human high-affinity choline transporter. FEBS letters *484*, 92-97.

Orjih, A.U., Banyal, H.S., Chevli, R., and Fitch, C.D. (1981). Hemin lyses malaria parasites. Science *214*, 667-669.

Ozarda Ilcol, Y., Uncu, G., and Ulus, I.H. (2002). Free and phospholipid-bound choline concentrations in serum during pregnancy, after delivery and in newborns. Archives of physiology and biochemistry *110*, 393-399.

Pachlatko, E., Rusch, S., Muller, A., Hemphill, A., Tilley, L., Hanssen, E., and Beck, H.P. (2010). MAHRP2, an exported protein of Plasmodium falciparum, is an essential component of Maurer's cleft tethers. Molecular microbiology 77, 1136-1152.

Pagola, S., Stephens, P.W., Bohle, D.S., Kosar, A.D., and Madsen, S.K. (2000). The structure of malaria pigment beta-haematin. Nature *404*, 307-310.

Painter, H.J., Morrisey, J.M., Mather, M.W., and Vaidya, A.B. (2007). Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. Nature *446*, 88-91.

Parys, J.B., Sernett, S.W., DeLisle, S., Snyder, P.M., Welsh, M.J., and Campbell, K.P. (1992). Isolation, characterization, and localization of the inositol 1,4,5-trisphosphate receptor protein in Xenopus laevis oocytes. The Journal of biological chemistry *267*, 18776-18782.

Patzewitz, E.M., Salcedo-Sora, J.E., Wong, E.H., Sethia, S., Stocks, P.A., Maughan, S.C., Murray, J.A., Krishna, S., Bray, P.G., Ward, S.A*., et al.* (2013). Glutathione transport: a new role for PfCRT in chloroquine resistance. Antioxidants & redox signaling *19*, 683-695.

Paul, F., Roath, S., Melville, D., Warhurst, D.C., and Osisanya, J.O. (1981). Separation of malaria-infected erythrocytes from whole blood: use of a selective high-gradient magnetic separation technique. Lancet 2, 70-71.

Pavlovic-Djuranovic, S., Kun, J.F., Schultz, J.E., and Beitz, E. (2006). Dihydroxyacetone and methylglyoxal as permeants of the Plasmodium aquaglyceroporin inhibit parasite proliferation. Biochimica et biophysica acta *1758*, 1012-1017.

Penarete-Vargas, D.M., Boisson, A., Urbach, S., Chantelauze, H., Peyrottes, S., Fraisse, L., and Vial, H.J. (2014). A chemical proteomics approach for the search of pharmacological targets of the antimalarial clinical candidate albitiazolium in Plasmodium falciparum using photocrosslinking and click chemistry. PloS one *9*, e113918.

Penny, J.I., Hall, S.T., Woodrow, C.J., Cowan, G.M., Gero, A.M., and Krishna, S. (1998). Expression of substrate-specific transporters encoded by Plasmodium falciparum in Xenopus laevis oocytes. Molecular and biochemical parasitology *93*, 81-89.

Pereira, M.R., Henrich, P.P., Sidhu, A.B., Johnson, D., Hardink, J., Van Deusen, J., Lin, J., Gore, K., O'Brien, C., Wele, M., et al. (2011). In vivo and in vitro antimalarial properties of azithromycin-chloroquine combinations that include the resistance reversal agent amlodipine. Antimicrobial agents and chemotherapy 55, 3115-3124.

Perez, D.I., Gil, C., and Martinez, A. (2011). Protein kinases CK1 and CK2 as new targets for neurodegenerative diseases. Medicinal research reviews 31, 924-954.

Perron-Savard, P., De Crescenzo, G., and Le Moual, H. (2005). Dimerization and DNA binding of the Salmonella enterica PhoP response regulator are phosphorylation independent. Microbiology *151*, 3979-3987.

Pesce, E.R., Acharya, P., Tatu, U., Nicoll, W.S., Shonhai, A., Hoppe, H.C., and Blatch, G.L. (2008). The Plasmodium falciparum heat shock protein 40, Pfj4, associates with heat shock protein 70 and shows similar heat induction and localisation patterns. The international journal of biochemistry & cell biology *40*, 2914-2926.

Pessi, G., Kociubinski, G., and Mamoun, C.B. (2004). A pathway for phosphatidylcholine biosynthesis in Plasmodium falciparum involving phosphoethanolamine methylation. Proceedings of the National Academy of Sciences of the United States of America *101*, 6206-6211.

Petersen, I., Gabryszewski, S.J., Johnston, G.L., Dhingra, S.K., Ecker, A., Lewis, R.E., de Almeida, M.J., Straimer, J., Henrich, P.P., Palatulan, E*., et al.* (2015). Balancing drug resistance and growth rates via compensatory mutations in the Plasmodium falciparum chloroquine resistance transporter. Molecular microbiology *97*, 381-395.

Peyrottes, S., Caldarelli, S., Wein, S., Perigaud, C., and Vial, H. (2014). Exploring prodrug approaches for albitiazolium and its analogues. Current topics in medicinal chemistry *14*, 1653-1667.

Pham, J.S., Sakaguchi, R., Yeoh, L.M., De Silva, N.S., McFadden, G.I., Hou, Y.M., and Ralph, S.A. (2014). A dual-targeted aminoacyl-tRNA synthetase in Plasmodium falciparum charges cytosolic and apicoplast tRNACys. The Biochemical journal *458*, 513-523.

Pisciotta, J.M., Coppens, I., Tripathi, A.K., Scholl, P.F., Shuman, J., Bajad, S., Shulaev, V., and Sullivan, D.J., Jr. (2007). The role of neutral lipid nanospheres in Plasmodium falciparum haem crystallization. The Biochemical journal *402*, 197-204.

Pochini, L., Scalise, M., Galluccio, M., Pani, G., Siminovitch, K.A., and Indiveri, C. (2012). The human OCTN1 (SLC22A4) reconstituted in liposomes catalyzes acetylcholine transport which is defective in the mutant L503F associated to the Crohn's disease. Biochimica et biophysica acta 1818, 559-565.

Ponts, N., Saraf, A., Chung, D.W., Harris, A., Prudhomme, J., Washburn, M.P., Florens, L., and Le Roch, K.G. (2011). Unraveling the ubiquitome of the human malaria parasite. The Journal of biological chemistry *286*, 40320-40330.

Preiser, P., Renia, L., Singh, N., Balu, B., Jarra, W., Voza, T., Kaneko, O., Blair, P., Torii, M., Landau, I*., et al.* (2004). Antibodies against MAEBL ligand domains M1 and M2 inhibit sporozoite development in vitro. Infection and immunity *72*, 3604-3608.

Price, R.N., and Nosten, F. (2014). Single-dose radical cure of Plasmodium vivax: a step closer. Lancet *383*, 1020-1021.

Price, R.N., Uhlemann, A.C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N.J*., et al.* (2004). Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet *364*, 438-447.

Prommana, P., Uthaipibull, C., Wongsombat, C., Kamchonwongpaisan, S., Yuthavong, Y., Knuepfer, E., Holder, A.A., and Shaw, P.J. (2013). Inducible knockdown of Plasmodium gene expression using the glmS ribozyme. PloS one *8*, e73783.

Prudencio, M., Rodriguez, A., and Mota, M.M. (2006). The silent path to thousands of merozoites: the Plasmodium liver stage. Nature reviews Microbiology 4, 849-856.

Pulcini, S., Staines, H.M., Pittman, J.K., Slavic, K., Doerig, C., Halbert, J., Tewari, R., Shah, F., Avery, M.A., Haynes, R.K*., et al.* (2013). Expression in yeast links field polymorphisms in PfATP6 to in vitro artemisinin resistance and identifies new inhibitor classes. The Journal of infectious diseases *208*, 468-478.

Pult, F., Fallah, G., Braam, U., Detro-Dassen, S., Niculescu, C., Laube, B., and Schmalzing, G. (2011). Robust post-translocational N-glycosylation at the extreme C-terminus of membrane and secreted proteins in Xenopus laevis oocytes and HEK293 cells. Glycobiology *21*, 1147-1160.

Raj, D.K., Mu, J., Jiang, H., Kabat, J., Singh, S., Sullivan, M., Fay, M.P., McCutchan, T.F., and Su, X.Z. (2009). Disruption of a Plasmodium falciparum multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione. The Journal of biological chemistry *284*, 7687-7696.

Rasoloson, D., Shi, L., Chong, C.R., Kafsack, B.F., and Sullivan, D.J. (2004). Copper pathways in Plasmodium falciparum infected erythrocytes indicate an efflux role for the copper P-ATPase. The Biochemical journal *381*, 803-811.

Razakantoanina, V., Florent, I., and Jaureguiberry, G. (2008). Plasmodium falciparum: functional mitochondrial ADP/ATP transporter in Escherichia coli plasmic membrane as a tool for selective drug screening. Experimental parasitology *118*, 181-187.

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K., and Cowman, A.F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature *403*, 906-909.

Richard, D., MacRaild, C.A., Riglar, D.T., Chan, J.A., Foley, M., Baum, J., Ralph, S.A., Norton, R.S., and Cowman, A.F. (2010). Interaction between Plasmodium falciparum apical membrane antigen 1 and the rhoptry neck protein complex defines a key step in the erythrocyte invasion process of malaria parasites. The Journal of biological chemistry *285*, 14815-14822.

Richter, J., Franken, G., Mehlhorn, H., Labisch, A., and Haussinger, D. (2010). What is the evidence for the existence of Plasmodium ovale hypnozoites? Parasitology research *107*, 1285-1290.

Riglar, D.T., Richard, D., Wilson, D.W., Boyle, M.J., Dekiwadia, C., Turnbull, L., Angrisano, F., Marapana, D.S., Rogers, K.L., Whitchurch, C.B.*, et al.* (2011). Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. Cell host & microbe *9*, 9-20.

Rivera, V.M., Wang, X., Wardwell, S., Courage, N.L., Volchuk, A., Keenan, T., Holt, D.A., Gilman, M., Orci, L., Cerasoli, F., Jr.*, et al.* (2000). Regulation of protein secretion through controlled aggregation in the endoplasmic reticulum. Science *287*, 826-830.

Roberts, L. (2016). Malaria wars. Science *352*, 398-402, 404-395.

Rodrigues, C.D., Hannus, M., Prudencio, M., Martin, C., Goncalves, L.A., Portugal, S., Epiphanio, S., Akinc, A., Hadwiger, P., Jahn-Hofmann, K.*, et al.* (2008). Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection. Cell host & microbe *4*, 271-282.

Rohrbach, P., Sanchez, C.P., Hayton, K., Friedrich, O., Patel, J., Sidhu, A.B., Ferdig, M.T., Fidock, D.A., and Lanzer, M. (2006). Genetic linkage of pfmdr1 with food vacuolar solute import in Plasmodium falciparum. The EMBO journal *25*, 3000-3011.

Roth, M., Obaidat, A., and Hagenbuch, B. (2012). OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. British journal of pharmacology *165*, 1260-1287.

Rotmann, A., Sanchez, C., Guiguemde, A., Rohrbach, P., Dave, A., Bakouh, N., Planelles, G., and Lanzer, M. (2010). PfCHA is a mitochondrial divalent cation/H+ antiporter in Plasmodium falciparum. Molecular microbiology *76*, 1591-1606.

Rottmann, M., McNamara, C., Yeung, B.K., Lee, M.C., Zou, B., Russell, B., Seitz, P., Plouffe, D.M., Dharia, N.V., Tan, J.*, et al.* (2010). Spiroindolones, a potent compound class for the treatment of malaria. Science *329*, 1175-1180.

Roux, K.J., Kim, D.I., Raida, M., and Burke, B. (2012). A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. The Journal of cell biology *196*, 801-810.

Rts, S.C.T.P. (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet *386*, 31-45.

Ruzzene, M., Penzo, D., and Pinna, L.A. (2002). Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. The Biochemical journal *364*, 41-47.

Sa, J.M., Twu, O., Hayton, K., Reyes, S., Fay, M.P., Ringwald, P., and Wellems, T.E. (2009). Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proceedings of the National Academy of Sciences of the United States of America *106*, 18883-18889.

Sabaty, M., Grosse, S., Adryanczyk, G., Boiry, S., Biaso, F., Arnoux, P., and Pignol, D. (2013). Detrimental effect of the 6 His C-terminal tag on YedY enzymatic activity and influence of the TAT signal sequence on YedY synthesis. BMC biochemistry *14*, 28.

Saitoh, M., Ishikawa, T., Matsushima, S., Naka, M., and Hidaka, H. (1987). Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. The Journal of biological chemistry *262*, 7796-7801.

Saliba, K.J., Horner, H.A., and Kirk, K. (1998). Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite Plasmodium falciparum. The Journal of biological chemistry *273*, 10190-10195.

Saliba, K.J., Martin, R.E., Broer, A., Henry, R.I., McCarthy, C.S., Downie, M.J., Allen, R.J., Mullin, K.A., McFadden, G.I., Broer, S*., et al.* (2006). Sodium-dependent uptake of inorganic phosphate by the intracellular malaria parasite. Nature *443*, 582-585.

Sanchez, C.P., Liu, C.H., Mayer, S., Nurhasanah, A., Cyrklaff, M., Mu, J., Ferdig, M.T., Stein, W.D., and Lanzer, M. (2014). A HECT ubiquitin-protein ligase as a novel candidate gene for altered quinine and quinidine responses in Plasmodium falciparum. PLoS genetics *10*, e1004382.

Sanchez, C.P., Mayer, S., Nurhasanah, A., Stein, W.D., and Lanzer, M. (2011). Genetic linkage analyses redefine the roles of PfCRT and PfMDR1 in drug accumulation and susceptibility in Plasmodium falciparum. Molecular microbiology *82*, 865-878.

Sanchez, C.P., McLean, J.E., Rohrbach, P., Fidock, D.A., Stein, W.D., and Lanzer, M. (2005). Evidence for a pfcrt-associated chloroquine efflux system in the human malarial parasite Plasmodium falciparum. Biochemistry *44*, 9862-9870.

Sanchez, C.P., McLean, J.E., Stein, W., and Lanzer, M. (2004). Evidence for a substrate specific and inhibitable drug efflux system in chloroquine resistant Plasmodium falciparum strains. Biochemistry *43*, 16365-16373.

Sanchez, C.P., Rohrbach, P., McLean, J.E., Fidock, D.A., Stein, W.D., and Lanzer, M. (2007). Differences in trans-stimulated chloroquine efflux kinetics are linked to PfCRT in Plasmodium falciparum. Molecular microbiology *64*, 407-420.

Sanchez, C.P., Rotmann, A., Stein, W.D., and Lanzer, M. (2008a). Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in Plasmodium falciparum. Molecular microbiology *70*, 786-798.

Sanchez, C.P., Stein, W., and Lanzer, M. (2003). Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in Plasmodium falciparum. Biochemistry *42*, 9383-9394.

Sanchez, C.P., Stein, W.D., and Lanzer, M. (2008b). Dissecting the components of quinine accumulation in Plasmodium falciparum. Molecular microbiology *67*, 1081-1093.

Sanofi Press Release (30 Oct 2013). Online: http://en.sanofi.com/Images/34723_20131030_Q32013_en.pdf

Saridaki, T., Sanchez, C.P., Pfahler, J., and Lanzer, M. (2008). A conditional export system provides new insights into protein export in Plasmodium falciparum-infected erythrocytes. Cellular microbiology *10*, 2483-2495.

Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatar, C., Pouvelle, B., Gysin, J., and Lanzer, M. (1998). Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. The EMBO journal *17*, 5418-5426.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B*., et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nature methods *9*, 676-682.

Schneider-Poetsch, T., Ju, J., Eyler, D.E., Dang, Y., Bhat, S., Merrick, W.C., Green, R., Shen, B., and Liu, J.O. (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nature chemical biology *6*, 209-217.

Sidhu, A.B., Uhlemann, A.C., Valderramos, S.G., Valderramos, J.C., Krishna, S., and Fidock, D.A. (2006). Decreasing pfmdr1 copy number in plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. The Journal of infectious diseases *194*, 528-535.

Sidhu, A.B., Valderramos, S.G., and Fidock, D.A. (2005). pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in Plasmodium falciparum. Molecular microbiology *57*, 913-926.

Sidhu, A.B., Verdier-Pinard, D., and Fidock, D.A. (2002). Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science *298*, 210-213.

Sijwali, P.S., Koo, J., Singh, N., and Rosenthal, P.J. (2006). Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of Plasmodium falciparum. Molecular and biochemical parasitology *150*, 96-106.

Silvie, O., Franetich, J.F., Charrin, S., Mueller, M.S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C.H.*, et al.* (2004). A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites. The Journal of biological chemistry *279*, 9490-9496.

Silvie, O., Rubinstein, E., Franetich, J.F., Prenant, M., Belnoue, E., Renia, L., Hannoun, L., Eling, W., Levy, S., Boucheix, C.*, et al.* (2003). Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. Nature medicine *9*, 93-96.

Sinclair, C.J., Chi, K.D., Subramanian, V., Ward, K.L., and Green, R.M. (2000). Functional expression of a high affinity mammalian hepatic choline/organic cation transporter. Journal of lipid research 41, 1841-1848.

Singh, S., Alam, M.M., Pal-Bhowmick, I., Brzostowski, J.A., and Chitnis, C.E. (2010). Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. PLoS pathogens *6*, e1000746.

Sinha, A., Hughes, K.R., Modrzynska, K.K., Otto, T.D., Pfander, C., Dickens, N.J., Religa, A.A., Bushell, E., Graham, A.L., Cameron, R.*, et al.* (2014). A cascade of DNA-binding proteins for sexual commitment and development in Plasmodium. Nature *507*, 253-257.

Sisowath, C., Petersen, I., Veiga, M.I., Martensson, A., Premji, Z., Bjorkman, A., Fidock, D.A., and Gil, J.P. (2009). In vivo selection of Plasmodium falciparum parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with artemether-lumefantrine in Africa. The Journal of infectious diseases *199*, 750-757.

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A., and Gil, J.P. (2005). In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). The Journal of infectious diseases *191*, 1014-1017.

Sive, H.L., Grainger, R.M., and Harland, R.M. (2010). Microinjection of RNA and preparation of secreted proteins from Xenopus oocytes. Cold Spring Harbor protocols *2010*, pdb prot5538.

Slavic, K., Krishna, S., Lahree, A., Bouyer, G., Hanson, K.K., Vera, I., Pittman, J.K., Staines, H.M., and Mota, M.M. (2016). A vacuolar iron-transporter homologue acts as a detoxifier in Plasmodium. Nature communications *7*, 10403.

Sleebs, B.E., Lopaticki, S., Marapana, D.S., O'Neill, M.T., Rajasekaran, P., Gazdik, M., Gunther, S., Whitehead, L.W., Lowes, K.N., Barfod, L.*, et al.* (2014). Inhibition of Plasmepsin V activity demonstrates its essential role in protein export, PfEMP1 display, and survival of malaria parasites. PLoS biology *12*, e1001897.

Smilkstein, M., Sriwilaijaroen, N., Kelly, J.X., Wilairat, P., and Riscoe, M. (2004). Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrobial agents and chemotherapy *48*, 1803-1806.

Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., Pinches, R., Newbold, C.I., and Miller, L.H. (1995). Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell *82*, 101-110.

Solyakov, L., Halbert, J., Alam, M.M., Semblat, J.P., Dorin-Semblat, D., Reininger, L., Bottrill, A.R., Mistry, S., Abdi, A., Fennell, C.*, et al.* (2011). Global kinomic and phospho-proteomic analyses of the human malaria parasite Plasmodium falciparum. Nature communications *2*, 565.

Spielmann, T., and Beck, H.P. (2000). Analysis of stage-specific transcription in plasmodium falciparum reveals a set of genes exclusively transcribed in ring stage parasites. Molecular and biochemical parasitology *111*, 453-458.

Spielmann, T., Fergusen, D.J., and Beck, H.P. (2003). etramps, a new Plasmodium falciparum gene family coding for developmentally regulated and highly charged membrane proteins located at the parasite-host cell interface. Molecular biology of the cell *14*, 1529-1544.

Staines, H.M., Alkhalil, A., Allen, R.J., De Jonge, H.R., Derbyshire, E., Egee, S., Ginsburg, H., Hill, D.A., Huber, S.M., Kirk, K.*, et al.* (2007). Electrophysiological studies of malaria parasite-infected erythrocytes: current status. International journal for parasitology *37*, 475-482.

Staines, H.M., Derbyshire, E.T., Slavic, K., Tattersall, A., Vial, H., and Krishna, S. (2010). Exploiting the therapeutic potential of Plasmodium falciparum solute transporters. Trends in parasitology *26*, 284-296.

Straimer, J., Lee, M.C., Lee, A.H., Zeitler, B., Williams, A.E., Pearl, J.R., Zhang, L., Rebar, E.J., Gregory, P.D., Llinas, M.*, et al.* (2012). Site-specific genome editing in Plasmodium falciparum using engineered zinc-finger nucleases. Nature methods *9*, 993-998.

Su, X., Kirkman, L.A., Fujioka, H., and Wellems, T.E. (1997). Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant P. falciparum in Southeast Asia and Africa. Cell *91*, 593-603.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K.J., Crisanti, A., Nussenzweig, V., Nussenzweig, R.S., and Menard, R. (1997). TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites. Cell *90*, 511-522.

Sullivan, D.J., Jr., Gluzman, I.Y., Russell, D.G., and Goldberg, D.E. (1996). On the molecular mechanism of chloroquine's antimalarial action. Proceedings of the National Academy of Sciences of the United States of America *93*, 11865-11870.

Sullivan, J.S., Sullivan, J.J., Williams, A., Grady, K.K., Bounngaseng, A., Huber, C.S., Nace, D., Williams, T., Galland, G.G., Barnwell, J.W.*, et al.* (2003). Adaptation of a strain of Plasmodium falciparum from Ghana to Aotus lemurinus griseimembra, A. nancymai, and A. vociferans monkeys. The American journal of tropical medicine and hygiene *69*, 593-600.

Summers, R.L., Dave, A., Dolstra, T.J., Bellanca, S., Marchetti, R.V., Nash, M.N., Richards, S.N., Goh, V., Schenk, R.L., Stein, W.D.*, et al.* (2014). Diverse mutational pathways converge on saturable chloroquine transport via the malaria parasite's chloroquine resistance transporter. Proceedings of the National Academy of Sciences of the United States of America *111*, E1759-1767.

Summers, R.L., and Martin, R.E. (2010). Functional characteristics of the malaria parasite's "chloroquine resistance transporter": implications for chemotherapy. Virulence *1*, 304-308.

Sutherland, C.J., Tanomsing, N., Nolder, D., Oguike, M., Jennison, C., Pukrittayakamee, S., Dolecek, C., Hien, T.T., do Rosario, V.E., Arez, A.P.*, et al.* (2010). Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. The Journal of infectious diseases *201*, 1544-1550.

Swamy, L., Amulic, B., and Deitsch, K.W. (2011). Plasmodium falciparum var gene silencing is determined by cis DNA elements that form stable and heritable interactions. Eukaryotic cell *10*, 530-539.

Tansley, R., Lotharius, J., Priestley, A., Bull, F., Duparc, S., and Mohrle, J. (2010). A randomized, double-blind, placebo-controlled study to investigate the safety, tolerability, and pharmacokinetics of single enantiomer (+)-mefloquine compared with racemic mefloquine in healthy persons. The American journal of tropical medicine and hygiene *83*, 1195-1201.

Tao, H., Liu, W., Simmons, B.N., Harris, H.K., Cox, T.C., and Massiah, M.A. (2010). Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS. BioTechniques *48*, 61-64.

Tay, C.L., Jones, M.L., Hodson, N., Theron, M., Choudhary, J.S., and Rayner, J.C. (2016). Study of Plasmodium falciparum DHHC palmitoyl-transferases identifies a role for PfDHHC9 in gametocytogenesis. Cellular microbiology.

Tewari, R., Dorin, D., Moon, R., Doerig, C., and Billker, O. (2005). An atypical mitogen-activated protein kinase controls cytokinesis and flagellar motility during male gamete formation in a malaria parasite. Molecular microbiology *58*, 1253-1263.

Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science 193, 673-675.

Umeda, D., Yamada, K., and Tachibana, H. (2008). H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) induces reduction of myosin regulatory light chain phosphorylation and inhibits cell proliferation. European journal of pharmacology *590*, 61-66.

Vaid, A., Ranjan, R., Smythe, W.A., Hoppe, H.C., and Sharma, P. (2010). PfPI3K, a phosphatidylinositol-3 kinase from Plasmodium falciparum, is exported to the host erythrocyte and is involved in hemoglobin trafficking. Blood *115*, 2500-2507.

Vaidya, A.B., Morrisey, J.M., Zhang, Z., Das, S., Daly, T.M., Otto, T.D., Spillman, N.J., Wyvratt, M., Siegl, P., Marfurt, J.*, et al.* (2014). Pyrazoleamide compounds are potent antimalarials that target Na+ homeostasis in intraerythrocytic Plasmodium falciparum. Nature communications *5*, 5521.

van Schalkwyk, D.A., Walden, J.C., and Smith, P.J. (2001). Reversal of chloroquine resistance in Plasmodium falciparum using combinations of chemosensitizers. Antimicrobial agents and chemotherapy 45, 3171-3174.

Wagner, C.A., Friedrich, B., Setiawan, I., Lang, F., and Broer, S. (2000). The use of Xenopus laevis oocytes for the functional characterization of heterologously expressed membrane proteins. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology *10*, 1-12.

Waller, K.L., McBride, S.M., Kim, K., and McDonald, T.V. (2008). Characterization of two putative potassium channels in Plasmodium falciparum. Malaria journal *7*, 19.

Waller, K.L., Muhle, R.A., Ursos, L.M., Horrocks, P., Verdier-Pinard, D., Sidhu, A.B., Fujioka, H., Roepe, P.D., and Fidock, D.A. (2003). Chloroquine resistance modulated in vitro by expression levels of the Plasmodium falciparum chloroquine resistance transporter. The Journal of biological chemistry *278*, 33593-33601.

Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R., and Carter, R. (1987). Genetic analysis of the human malaria parasite Plasmodium falciparum. Science *236*, 1661-1666.

Wang, L., Delahunty, C., Prieto, J.H., Rahlfs, S., Jortzik, E., Yates, J.R., 3rd, and Becker, K. (2014). Protein S-nitrosylation in Plasmodium falciparum. Antioxidants & redox signaling *20*, 2923-2935.

Wang, X., Mu, J., Li, G., Chen, P., Guo, X., Fu, L., Chen, L., Su, X., and Wellems, T.E. (2005). Decreased prevalence of the Plasmodium falciparum chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against P. falciparum malaria in Hainan, People's Republic of China. The American journal of tropical medicine and hygiene *72*, 410-414.

Ward, P., Equinet, L., Packer, J., and Doerig, C. (2004). Protein kinases of the human malaria parasite Plasmodium falciparum: the kinome of a divergent eukaryote. BMC genomics *5*, 79.

Wein, S., Maynadier, M., Bordat, Y., Perez, J., Maheshwari, S., Bette-Bobillo, P., Tran Van Ba, C., Penarete-Vargas, D., Fraisse, L., Cerdan, R*., et al.* (2012). Transport and pharmacodynamics of albitiazolium, an antimalarial drug candidate. British journal of pharmacology *166*, 2263-2276.

Wein, S., Tran Van Ba, C., Maynadier, M., Bordat, Y., Perez, J., Peyrottes, S., Fraisse, L., and Vial, H.J. (2014). New insight into the mechanism of accumulation and intraerythrocytic compartmentation of albitiazolium, a new type of antimalarial. Antimicrobial agents and chemotherapy *58*, 5519-5527.

Wells, T.N., Hooft van Huijsduijnen, R., and Van Voorhis, W.C. (2015). Malaria medicines: a glass half full? Nature reviews Drug discovery *14*, 424-442.

World Health Organization (2014). Severe malaria. Tropical medicine & international health : TM & IH *19 Suppl 1*, 7-131.

World Health Organization (2015a). World Malaria Report 2015. Online: http://www.who.int/malaria/publications/world-malaria-report-2015/report/en

World Health Organization (2015b). Guidelines for the treatment of malaria. Third edition. April 2015. Online: http://www.who.int/malaria/publications/atoz/9789241549127/en

Wilkes, J.M., and Doerig, C. (2008). The protein-phosphatome of the human malaria parasite Plasmodium falciparum. BMC genomics *9*, 412.

Woo, J.S., Zeltina, A., Goetz, B.A., and Locher, K.P. (2012). X-ray structure of the Yersinia pestis heme transporter HmuUV. Nature structural & molecular biology 19, 1310-1315.

Woodrow, C.J., Penny, J.I., and Krishna, S. (1999). Intraerythrocytic Plasmodium falciparum expresses a high affinity facilitative hexose transporter. The Journal of biological chemistry *274*, 7272-7277.

Wootton, J.C., Feng, X., Ferdig, M.T., Cooper, R.A., Mu, J., Baruch, D.I., Magill, A.J., and Su, X.Z. (2002). Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. Nature *418*, 320-323.

Wright, M.H., Clough, B., Rackham, M.D., Rangachari, K., Brannigan, J.A., Grainger, M., Moss, D.K., Bottrill, A.R., Heal, W.P., Broncel, M*., et al.* (2014). Validation of N-myristoyltransferase as an antimalarial drug target using an integrated chemical biology approach. Nature chemistry *6*, 112-121.

Wu, Y., Kirkman, L.A., and Wellems, T.E. (1996). Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proceedings of the National Academy of Sciences of the United States of America *93*, 1130-1134.

Wu, Y., Sifri, C.D., Lei, H.H., Su, X.Z., and Wellems, T.E. (1995). Transfection of Plasmodium falciparum within human red blood cells. Proceedings of the National Academy of Sciences of the United States of America 92, 973-977.

Yamaki, T., Tanaka, T., and Hidaka, H. (1979). [Platelet cyclic nucleotides and calcium]. Rinsho byori The Japanese journal of clinical pathology *Suppl 40*, 67-73.

Yayon, A., Cabantchik, Z.I., and Ginsburg, H. (1984). Identification of the acidic compartment of Plasmodium falciparum-infected human erythrocytes as the target of the antimalarial drug chloroquine. The EMBO journal *3*, 2695-2700.

Yeoh, S., O'Donnell, R.A., Koussis, K., Dluzewski, A.R., Ansell, K.H., Osborne, S.A., Hackett, F., Withers-Martinez, C., Mitchell, G.H., Bannister, L.H.*, et al.* (2007). Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. Cell *131*, 1072-1083.

Yuthavong, Y., Tarnchompoo, B., Vilaivan, T., Chitnumsub, P., Kamchonwongpaisan, S., Charman, S.A., McLennan, D.N., White, K.L., Vivas, L., Bongard, E.*, et al.* (2012). Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. Proceedings of the National Academy of Sciences of the United States of America *109*, 16823-16828.

Zhang, C., Xiao, B., Jiang, Y., Zhao, Y., Li, Z., Gao, H., Ling, Y., Wei, J., Li, S., Lu, M.*, et al.* (2014). Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. mBio *5*, e01414-01414.

Zhang, J., Krugliak, M., and Ginsburg, H. (1999). The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. Molecular and biochemical parasitology *99*, 129-141.

Zhang, L., Dresser, M.J., Gray, A.T., Yost, S.C., Terashita, S., and Giacomini, K.M. (1997). Cloning and functional expression of a human liver organic cation transporter. Molecular pharmacology *51*, 913-921.

Zhang, L., Gorset, W., Washington, C.B., Blaschke, T.F., Kroetz, D.L., and Giacomini, K.M. (2000). Interactions of HIV protease inhibitors with a human organic cation transporter in a mammalian expression system. Drug metabolism and disposition: the biological fate of chemicals 28, 329-334.

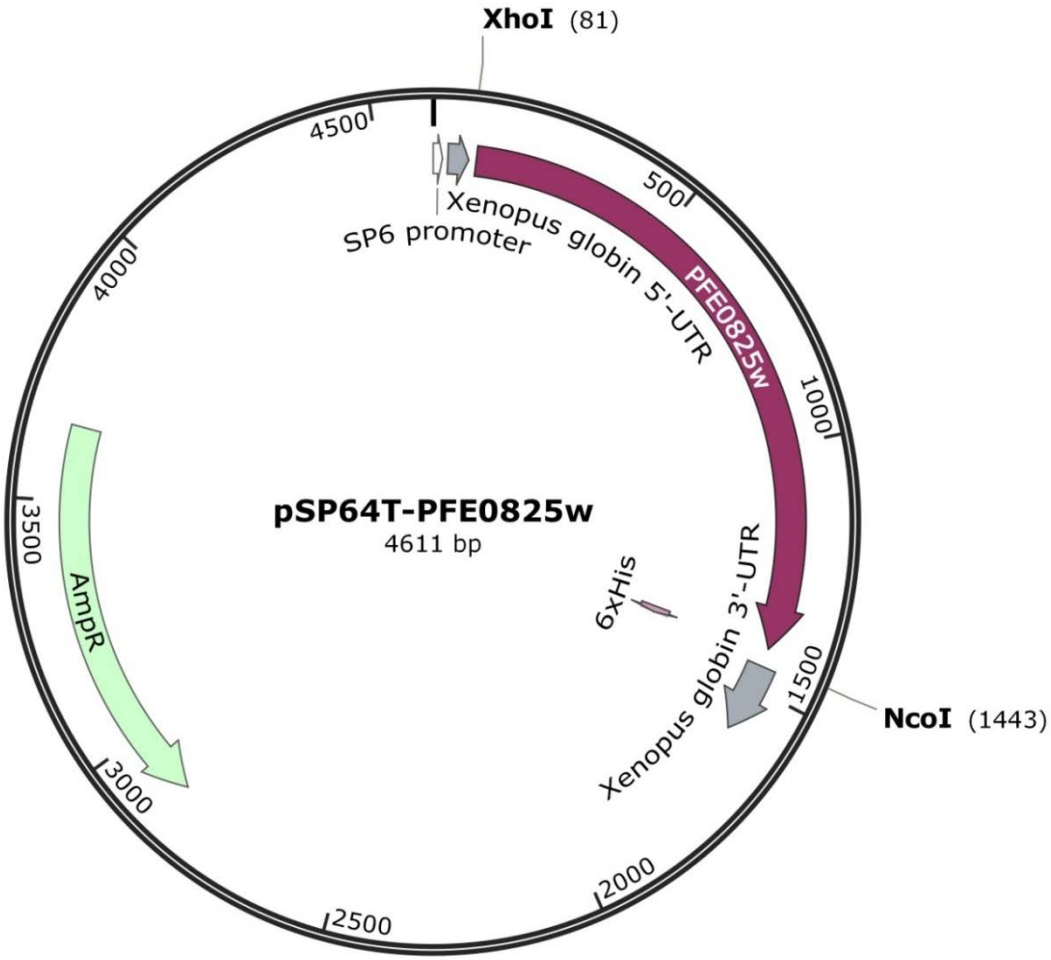
Zhang, M., Fennell, C., Ranford-Cartwright, L., Sakthivel, R., Gueirard, P., Meister, S., Caspi, A., Doerig, C., Nussenzweig, R.S., Tuteja, R.*, et al.* (2010). The Plasmodium eukaryotic initiation factor-2alpha kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. The Journal of experimental medicine *207*, 1465-1474.

Zhang, M., Mishra, S., Sakthivel, R., Rojas, M., Ranjan, R., Sullivan, W.J., Jr., Fontoura, B.M., Menard, R., Dever, T.E., and Nussenzweig, V. (2012). PK4, a eukaryotic initiation factor 2alpha(eIF2alpha) kinase, is essential for the development of the erythrocytic cycle of Plasmodium. Proceedings of the National Academy of Sciences of the United States of America *109*, 3956-3961.

Zimmer, M., and Hofmann, F. (1984). Calmodulin antagonists inhibit activity of myosin light-chain kinase independent of calmodulin. European journal of biochemistry / FEBS *142*, 393-397.

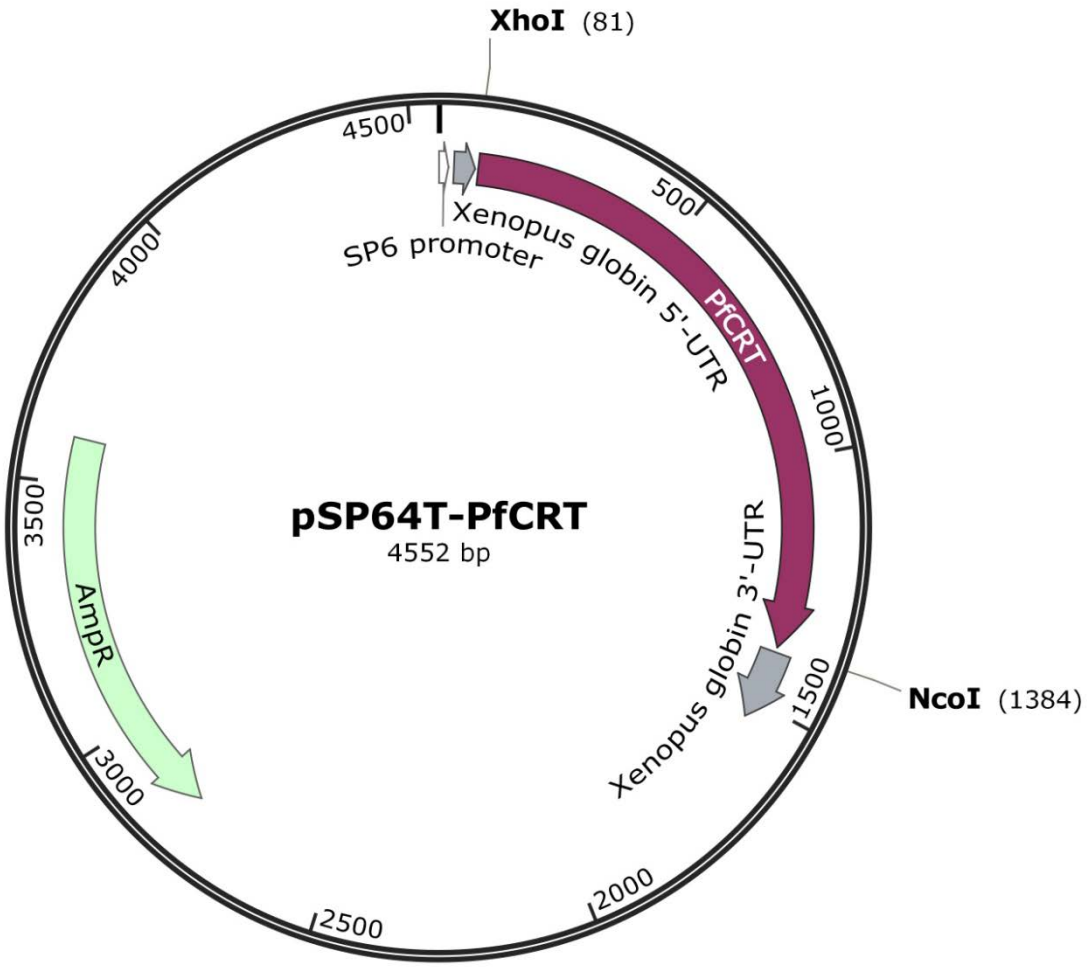
Appendix I: Plasmid maps

> pSP64T-*pfe0825w*



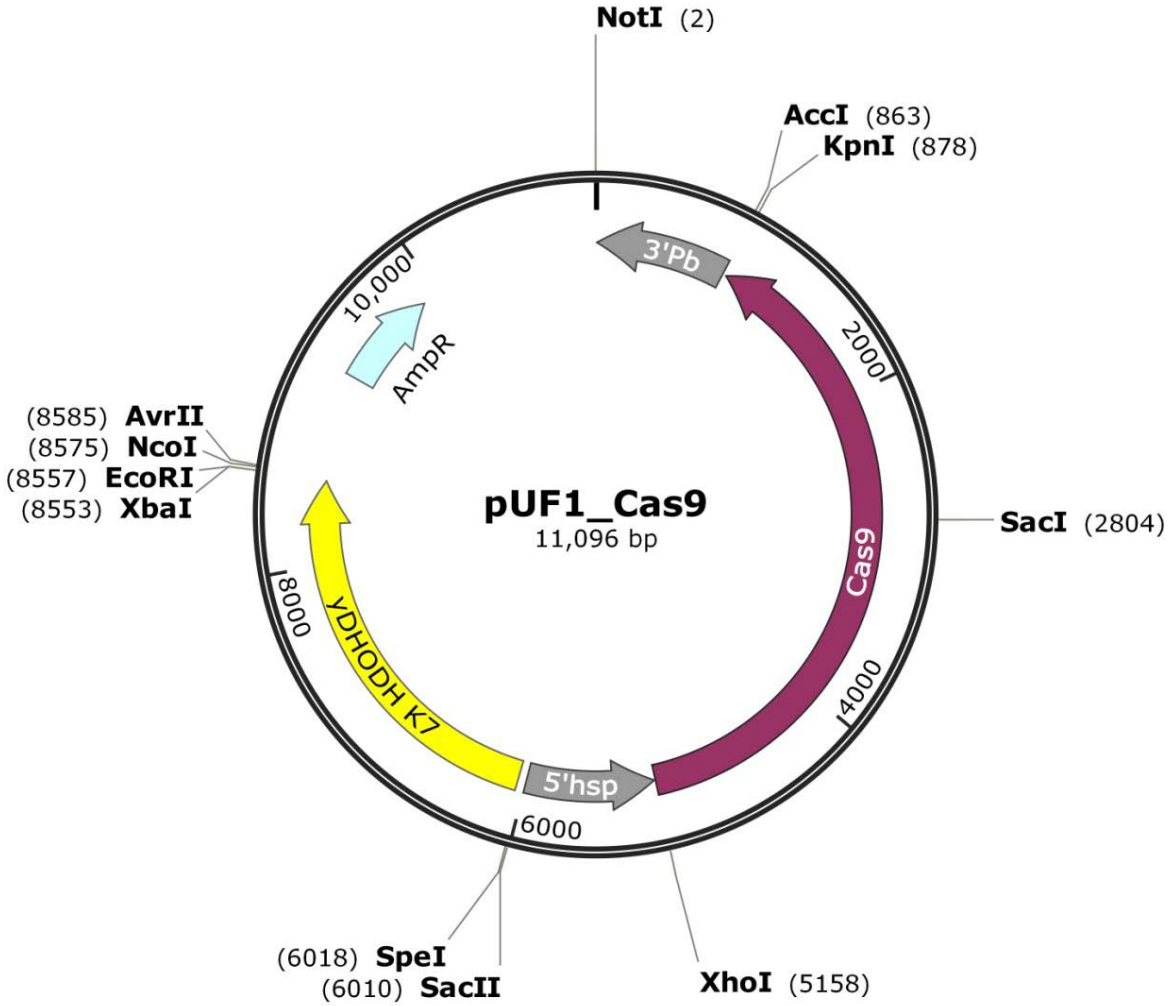
The sequences of the different *pfe0825w* variants were amplified by PCR from the codon adapted sequence of the gene (synthesized by GeneArt) including (primers 17-18-19) or not (primers 17-19-22) the 6x histidine tag. They were cloned into the pSP64T vector using the restriction sites XhoI and NcoI. The coding region of variant 2 that is not in frame in the other variants was cloned from *P. falciparum* gDNA and was not codon optimized (primers 19-21). *Pfe0825w* mRNA *in vitro* transcription is driven by the SP6 promoter. The *Pfe0825w* gene is flanked by the 5'UTR and 3'UTR regions of the *X. laevis* globin gene. The selection marker of the plasmid is the ampicillin resistance gene (*AmpR*).

> pSP64T-PfCRT^{Dd2}



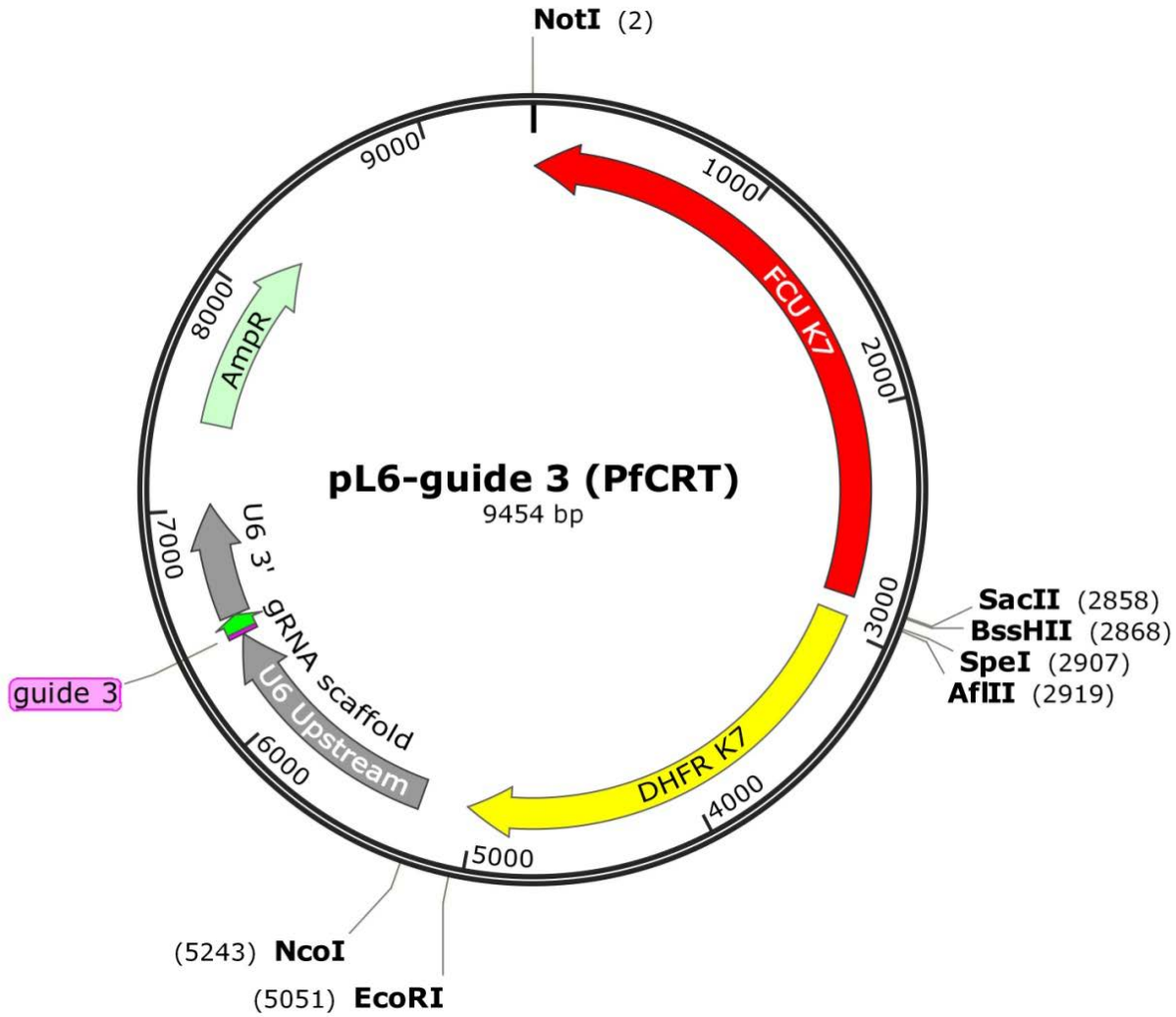
The sequence of PfCRT from the Dd2 strain was amplified by PCR from the codon adapted sequence of the gene (synthesized by GeneArt). It was cloned into the pSP64T vector using the restriction sites XhoI and NcoI. PfCRT mRNA *in vitro* transcription is driven by the SP6 promoter. The *pfcrt* gene is flanked by the 5'UTR and 3'UTR regions of the *X. laevis* globin gene. The selection marker of the plasmid is the ampicillin resistance gene (*AmpR*). This construct was provided by Dr. Sebastiano Bellanca.

> pUF1-Cas9



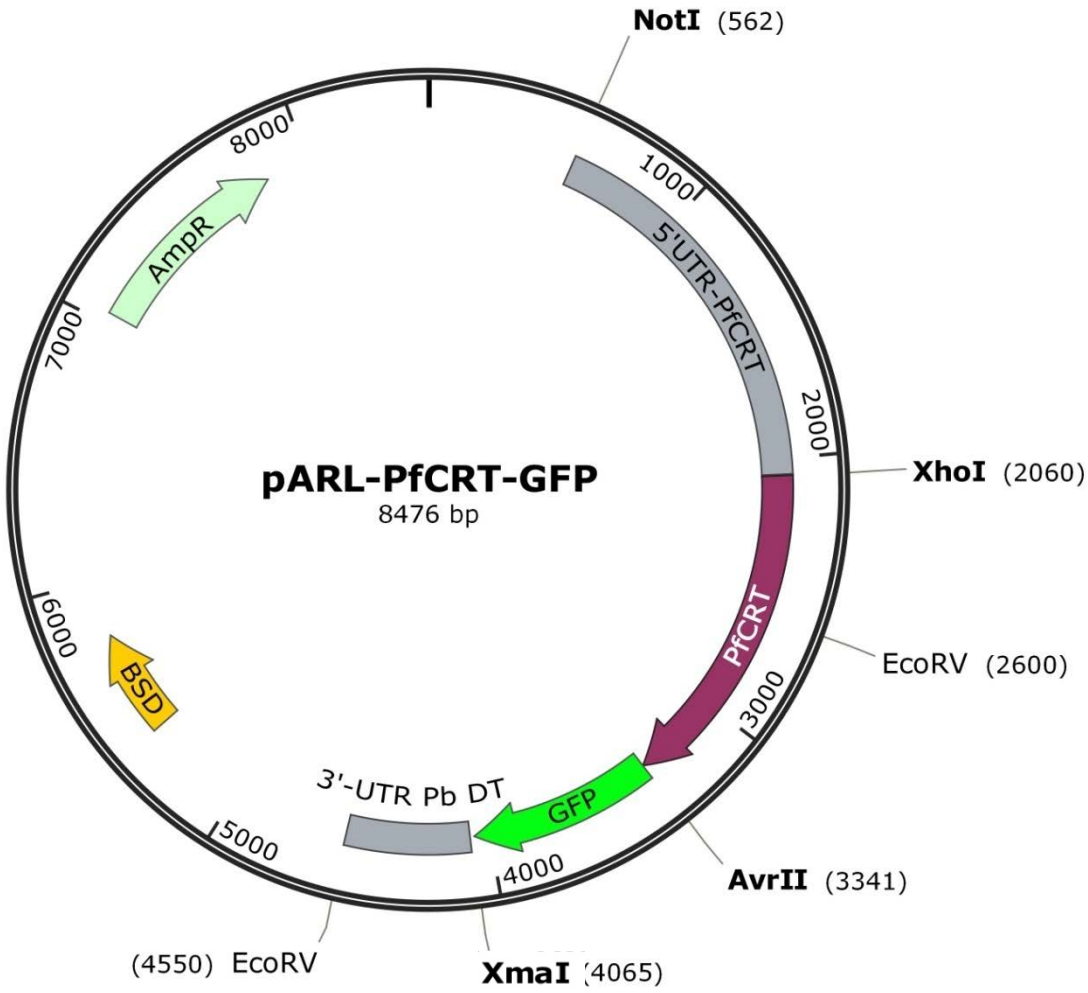
The vector pUF1-Cas9 codes for the sequence of the *Cas9* endonuclease flanked by nuclear localization signals (NLS). Its expression is regulated by the promoter region of the heat shock protein 86 (5' hsp) and the 3'UTR region of the *P. berghei* dhfr (3' Pb dhfr). The selection marker of the plasmid is the yeast dihydroorotate dehydrogenase gene (*ydhodh*). This plasmid was provided by Dr. José Juan Lopez Rubio (Ghorbal et al., 2014).

> pL6-guide 3 (PfCRT)



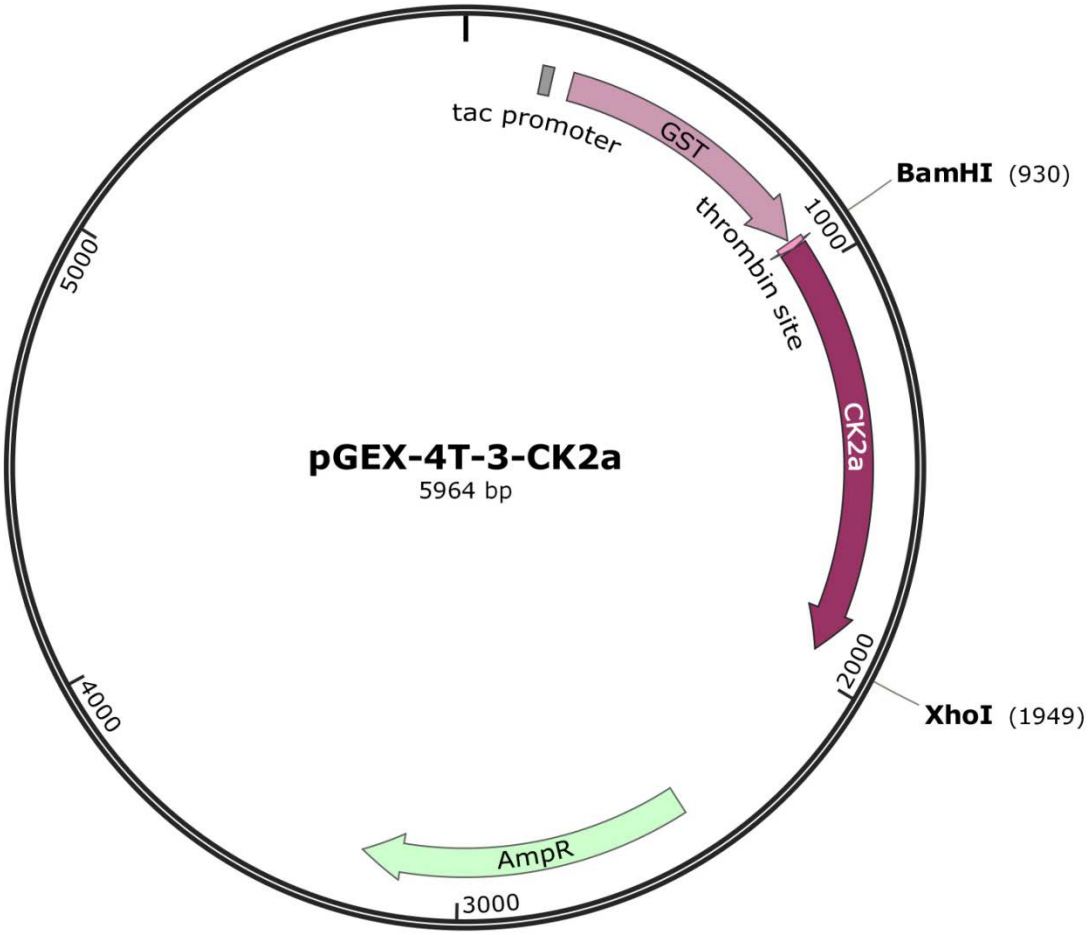
The pL6 plasmid contains the sgRNA-expression cassette. The expression of the sgRNA is regulated by the promoter and the 3'UTR region of the *P. falciparum* U6 snRNA polymerase III (5' U6). The selection marker of the plasmid is the human dihydrofolate reductase gene (*hdhfr*) and the negative selection marker is the bifunctional yeast cytosine deaminase and uridyl phosphoribosyl transferase (*yfcu*). The original pL6 vector was digested with the enzyme BtgZI and the PfCRT guide 3 sequence was cloned into the vector using the *In Fusion* cloning technology (primers 31-32). The original pL6 vector was provided by Dr. José Juan Lopez Rubio (Ghorbal et al., 2014).

>pARL-PfCRT^{Dd2}-GFP



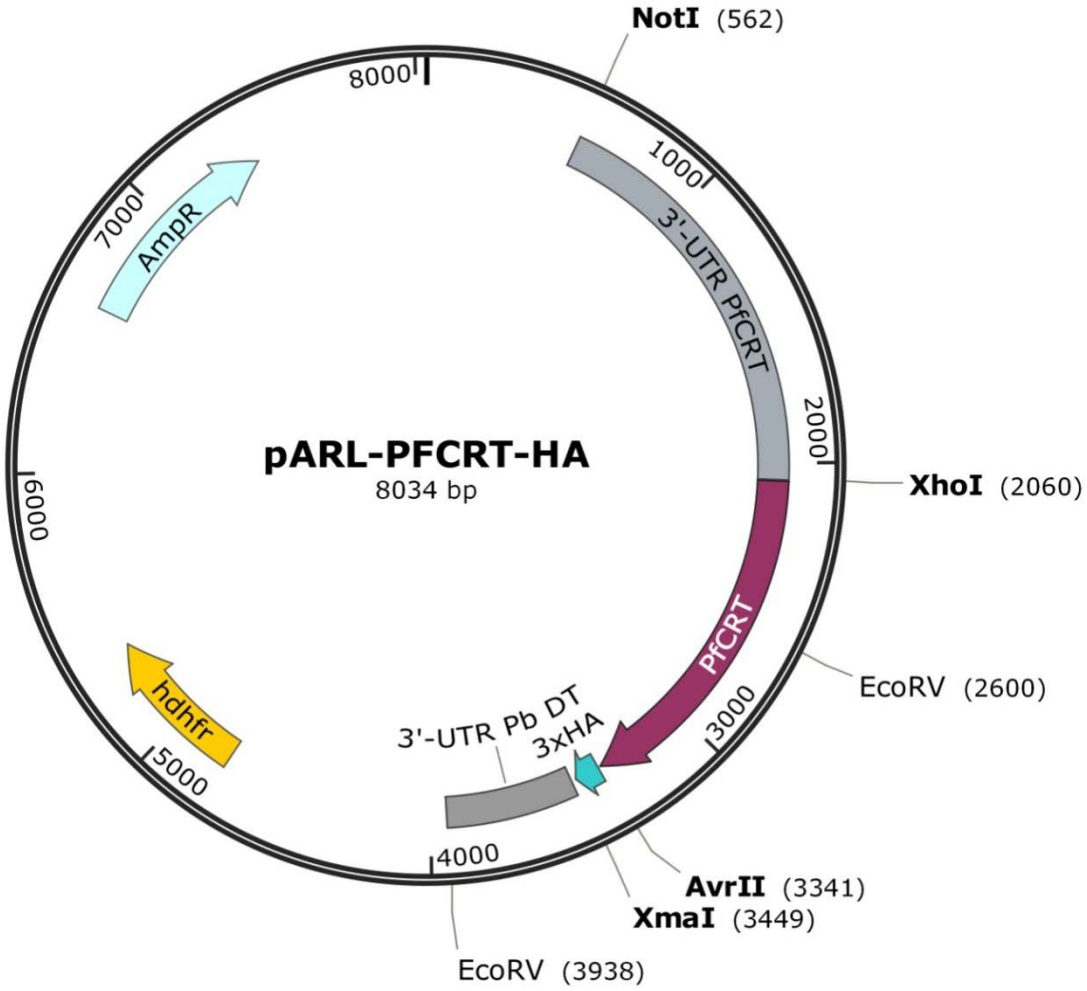
The PfCRT^{Dd2}-GFP sequence from a pARL-PfCRT^{Dd2}-GFP-hdhfr vector (provided by Dr. Cecilia Sanchez) was cloned into a pARL-BSD vector (provided by Dr. Sophia Deil) using the enzymes XhoI and XmaI. The expression of PfCRT is regulated by the PfCRT promoter and the 3'UTR region of the *P. berghei* DT. The original pARL1a^{+} vector was provided by Prof. Tim Gilberger (Crabb et al., 2004).

> pGEG-4T-3 GST, PfCK2α-GST and PfCK2α^{K72M}-GST



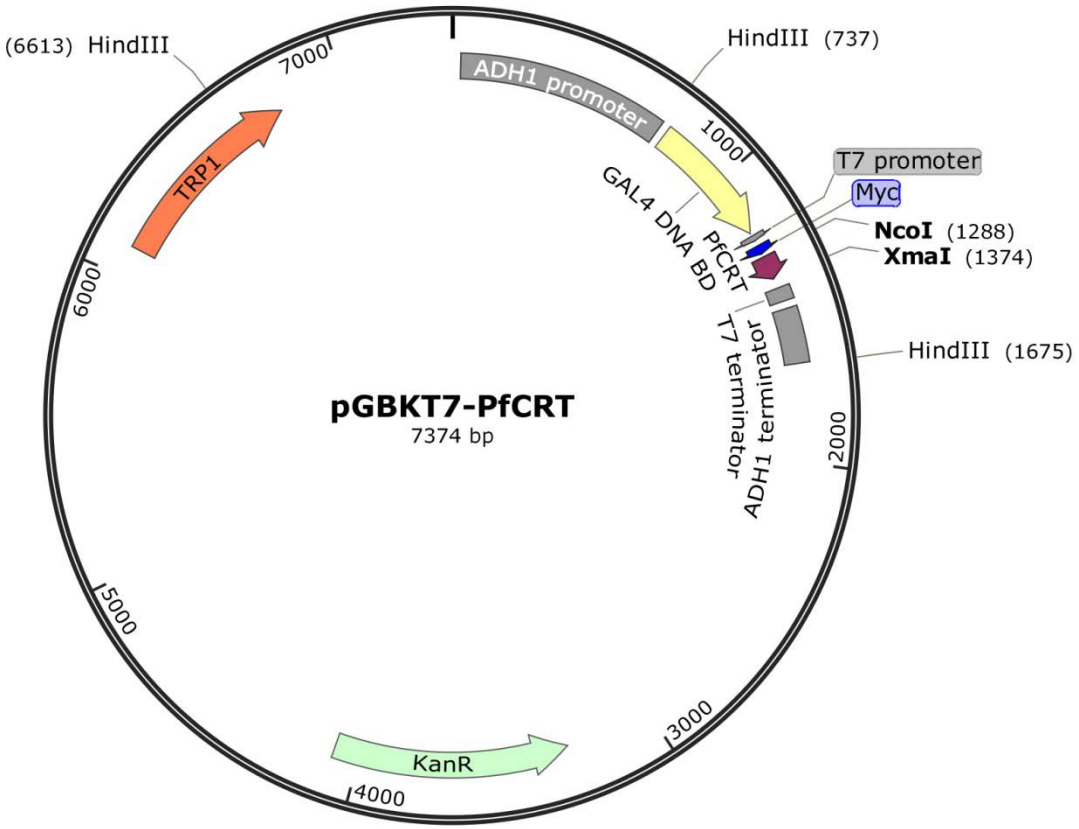
The pGEX-4T-3 is a commercially available plasmid from GE Healthcare Life Sciences. It is used to express recombinant proteins fused to GST in bacteria under the tac promoter (a hybrid promoter derived from the trp and lac promoters). It contains the resistance cassette for ampicillin and a thrombin cleavage site between the GST tag and the multicloning site. CK2α and CK2α^{K72M} sequences were amplified from *P. falciparum* 3D7 cDNA and cloned into the vector using the BamHI and XhoI restriction sites. Both plasmids were provided by Prof. Christian Doerig (Holland et al., 2009).

>pARL-PfCRT^{Dd2}-HA



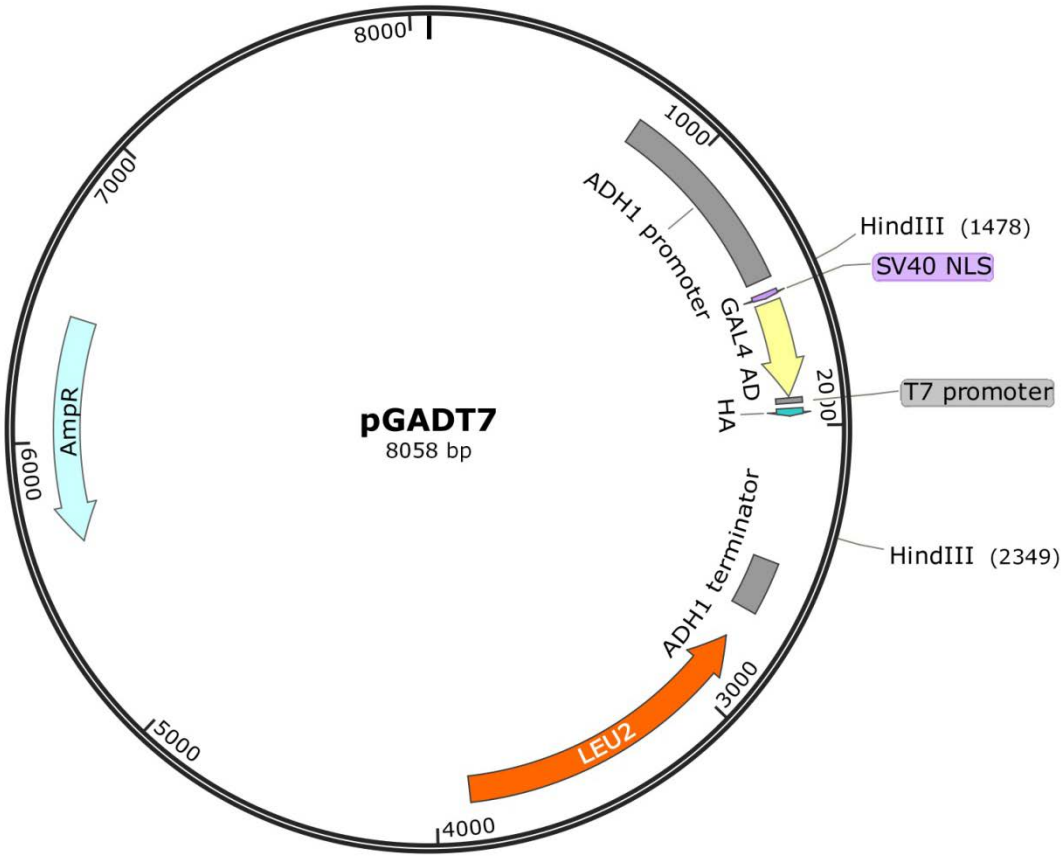
The GFP sequence from a pARL-PfCRT^{Dd2}-GFP construct was substituted by a 3xHis tag using the enzymes AvrII and XmaI. The expression of PfCRT is regulated by the PfCRT promoter and by the 3'UTR region of the *P. berghei* DT. The selection marker of the plasmid is the human dihydrofolate reductase gene (*hdhfr*). This construct was provided by Sarah Klinnert. The original pARL1a^{+} vector was provided by Prof. Tim Gilberger (Crabb et al., 2004).

> pGBKT7



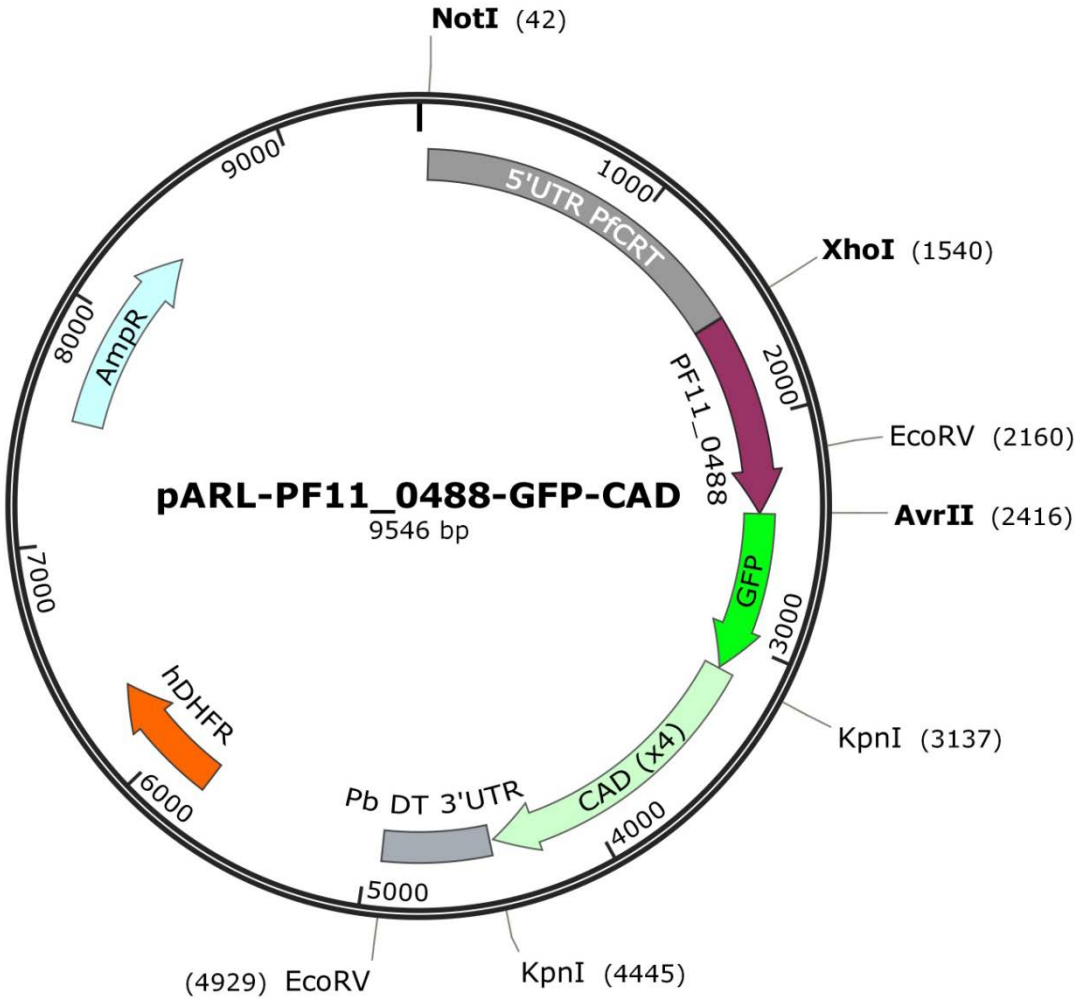
The pGBKT7 vector is a commercially available plasmid from Clontech. It is used to express recombinant proteins fused to the GAL4 DNA binding domain and the myc tag in yeast. The recombinant proteins are expressed in yeast under the control of the ADH1 promoter and the T7 and ADH1 terminators. The recombinant proteins fused to the myc tag can also be expressed *in vitro* from the T7 promoter. The plasmid contains the kanamicin resistance gene for selection in bacteria and the tryptophan nutritional marker for selection in yeast. The PfCRT sequences used as bait in the Y2H screen were amplified from *P. falciparum* Dd2 cDNA and cloned into de vector using the NcoI and XmaI restriction sites. All the constructs in the pGBKT7 vector were provided by Anne Christin Roth.

> pGADT7



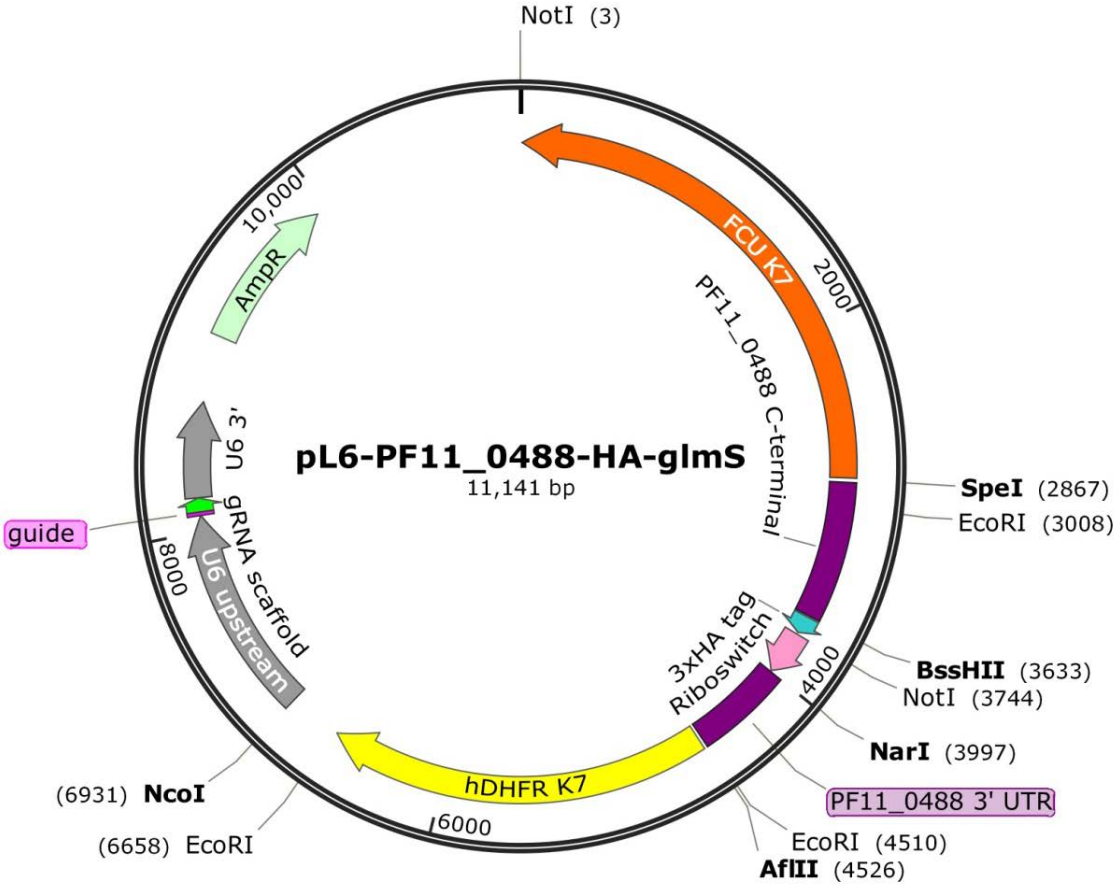
The pGADT7 vector is a commercially available plasmid from Clontech. It is used to express recombinant proteins fused to the GAL4 activation domain and the HA tag in yeast. It also contains the SV40 nuclear localization signal in order to target the proteins to the yeast nucleus. The recombinant proteins are expressed in yeast under the control of the ADH1 promoter and terminator. The recombinant proteins fused to the HA tag can also be expressed *in vitro* from the T7 promoter. The plasmid contains the ampicillin resistance gene for selection in bacteria and the leucine nutritional marker for selection in yeast. The plasmid was used to clone a cDNA library from the *P. falciparum* strain Dd2.

> pARL-PF11_0888 $^{C\text{-terminal}}$ -GFP-CAD



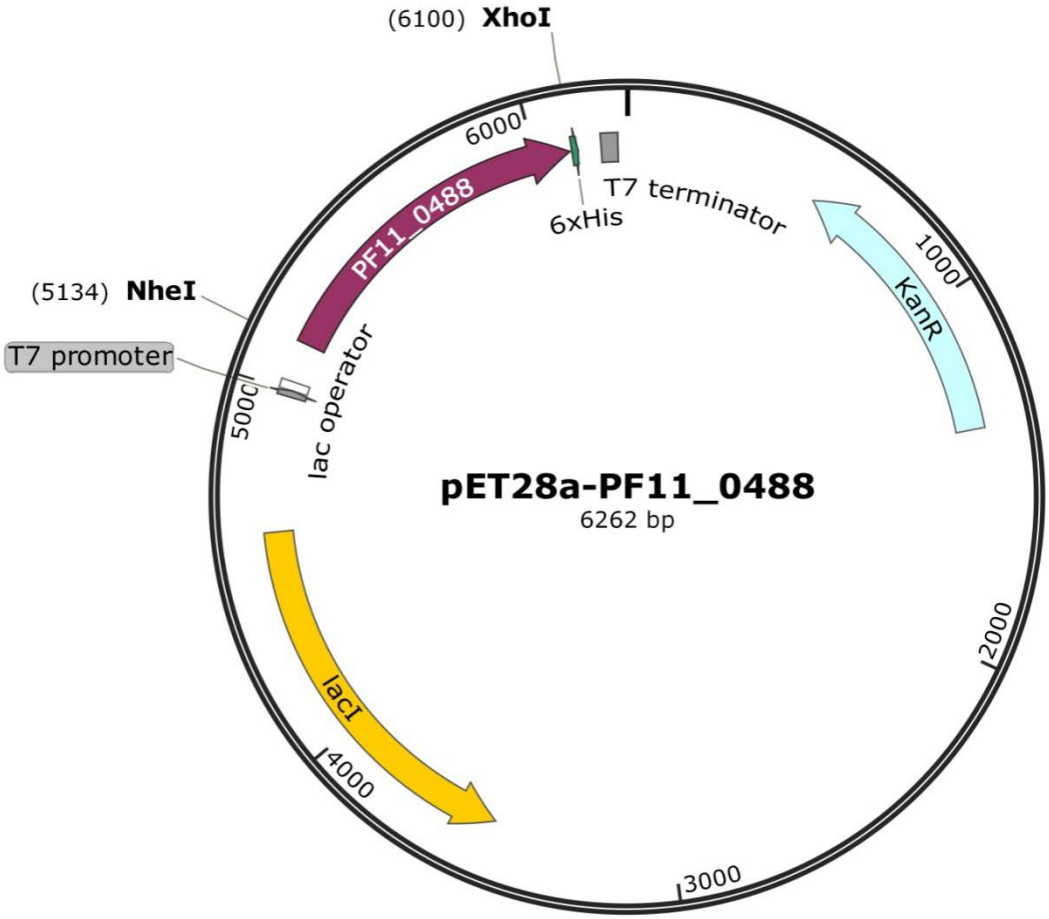
The *PF11_0488* C-terminal sequence (residues 1108-1429) was amplified from Dd2 cDNA and cloned into the pARL1a^{+} vector fused to GFP-CAD using the enzymes XhoI and AvrII. The expression of PF11_0488 $^{C\text{-terminal}}$ is regulated by the PfCRT promoter and by the 3'UTR region of the *P. berghei* DT. The selection marker of the plasmid is the human dihydrofolate reductase gene (*hdhfr*). This construct was provided by Dr. Cecilia Sanchez. The original pARL1a^{+} vector was provided by Prof. Tim Gilberger (Crabb et al., 2004).

> pL6-PF11_0888-HA-glmS



The pL6 plasmid contains the sgRNA-expression cassette. The expression of the sgRNA is regulated by the promoter and the 3'UTR region of the *P. falciparum* U6 snRNA polymerase III (5' U6). The selection marker of the plasmid is the human dihydrofolate reductase gene (*hdhfr*) and the negative selection marker is the bifunctional yeast cytosine deaminase and uridyl phosphoribosyl transferase (*yfcu*). The original pL6 vector was digested with the enzyme BtgZI and the PF11_0488 guide 1 (primers 44-45) or guide 2 (primers 46-47) sequences were cloned into the vector using the *In Fusion* cloning technology. A *PF11_04488* homology region from the C-terminal part of the gene (3529-4289 bp; primers 37-38) was cloned before the HA-glmS tag using the enzymes SpeI and BssHII. A 3'UTR homology region (1-523 bp; primers 42-43) was cloned after the HA-glmS tag using the enzymes NarI and AflII. The shield mutations were introduced by PCR (primers 39-40-41). The original pL6 vector was provided by Dr. José Juan Lopez Rubio.

> pET28a-PF11_0488$^{C\text{-terminal}}$



The pET28a vector is a commercially available plasmid from Novagen. It is used to express recombinant proteins fused to a 6xHis tag in *E.coli*. The recombinant proteins are expressed under the control of the T7 promoter and terminator and the lac operator. The expression can be induced by the addition of IPTG, which binds to the lac repressor (*lacI*). The plasmid contains the kanamicin resistance gene for selection in bacteria. The C-terminal sequence of *PF11_0488* (3328-4289 bp) was amplified from Dd2 cDNA and cloned into the pET28a vector using the restriction enzymes XhoI and NheI. This construct was provided by Anne Christin Roth.

Appendix II: DNA/Protein sequences

1. *PF11_0825w* alignment between *Plasmodium* species

P. vivax	PVX_080425
P. falciparum	PF3D7_0516500
P. chabaudi	PCHAS_1231900
P. berghei	PBANKA_1231300
P. yoelii	PY17X_1234700

```
P.vivax          MDVTSTLLDKSDSVAGDPSDAVPGAKKFFFSSIGKAHMINVLYGVGYTVQIAMLPYMLIS
P.falciparum     MEVTSTLLEKGKNFAQDPSEVFPESKKFFFSSIGKAHLINSLYGIGYTIQIAMLPYLLIS
P.chabaudi       MEVTSTLLQKSQMFADDSSDGFPTTKKFFVSSIGKAHLINSLYGIGYTIQIAMLPYLLIS
P.berguei        MEVTSTLLKKSQMFADDSSDGFPTTKKFFVSSIGKAHLINSLYGIGYTIQIAMLPYLLIN
P.yoelii         MEVTSTLLKKSQMFADDSSDGFPTTKKFFVSSIGKAHLINSLYGIGYTIQIAMLPYLLIN
                 *:******.*.. .* * *: .* :****.*******:** ***:***:*******:**.

P.vivax          SGAGIEHNGYLLTLFSLLQFVGSTFFGRLADIWGVKKSFYLSLCSSSLMYLMLPACRATW
P.falciparum     SNAGIEHNGYLLTLFSLLQFTGSIFFGRMADIWGVKKSFYLSLISSCLMYLMIMVCESTW
P.chabaudi       SNAGIEHNGYLLTLFSLLQFIGSIFFGRIADIWGVKKSFYLSLVSSSMMYLMLTISKSML
P.berguei        SNAGIEHNGYLLTLFSLLQFVGSIFFGRIADIWGVKKSFYLSLLSSSMMYLMLTVCRSVL
P.yoelii         SKAGIEHNGYLLTLFSLLQFIGSIFFGRIADIWGVKRSFYLSLVSSSIMYLMLTVCRSVL
                 * ****************** ** ****:*******:****** **.:****:  ..:

P.vivax          AYYVSFLPSFFMQTFQASSLLVCLKTDSEKRTAAIGYLNLSYGMGIILGSLIAGLMVNYV
P.falciparum     AYYISFLPSFFMQTFQASSLLVCLKTNFDKRTGALGYLNLSYGMGIIFGSFLAGVMVNFV
P.chabaudi       GYYISFVPSFFMQTFQVSSLLVCLKTENDNRTAAIGYLNLSYGIGIIFGSILAGMLVNIL
P.berguei        GYYISFFPSFFMQTFQVSSLLVCLKTENDKRTAAIGYLNLSYGIGIIMGSILAGMLVNIL
P.yoelii         GYYISFFPSFFMQTFQVSSLLVCLKTENDKRTAAIGYLNLSYGIGIIMGSILAGMLVNIL
                 .**:**.*********.*********: ::**.*:********:***:**::**::** :

P.vivax          GPRGNLLIALGSQIAALYVAKTLSEDPKLLKPVNLGDIKMREILSSIQNEYARVLNLFRK
P.falciparum     GSRGNLLIALLSQLIALCISTTLEEDPKLLKSSNVDKMKMSEILLSIKNEYIRVLNLFKK
P.chabaudi       GPRGNLFVAFLSQVLALYISKSLEEDPKLLVNNNIEKIKLKDLFTTAQNECTRLFKLFQK
P.berguei        GPKGNLFVAFLSQILALYISKSLEEDPKLLINNNIEKIKLKELFKTAQNECTRLFKLFKK
P.yoelii         GPRGNLFVAFLSQILALYISKNLEEDPKLLINNNIEKIKLKEIFKTAQNECTRLFKLFKK
                 * :***::*: **: ** ::..*.******   *: .:*: ::: : :**  *:::**:*

P.vivax          TYGMCLLILFGLLPILMTKFAFAPVVVDMFKLTPSHTSYLMTYAGIVTIIAEGLLAPYLS
P.falciparum     TYGICLLILFGLLPILMTKFAFAPVVVDMFKLTPSHTSYLMTYAGIITIIAEGILAPYLS
P.chabaudi       TYGICLLIIFGLLPILMTKFAFAPVVVDMFKLTPAHTSYLMTYAGIITIIAEGLLAPYLS
P.berguei        TYGICLLILFGLLPILMTKFAFAPVVVDMFKLTPAHTSYLMTYAGIITIIAEGLLAPYLS
P.yoelii         TYGICLLILFGLLPILMTKFAFAPVVVDMFKLTPAHTSYLMTYAGIITIIAEGLLAPYLS
                 ***:****:************************:***********:******:******

P.vivax          SILGDITCCKYSVPLTLAGFLALSLCGANEYLVLLFMSIPLCGGALLYICGTSQMTKRVE
P.falciparum     SLLGDMICCKYSIPLTLTGFLLLSLCGANESLVLIFMSIPLCGGALLYICGTSQMTKRVE
P.chabaudi       TLLGDMICCKFAIPLTLSGFLLLSLCGANEMFVLLFMIIPLCGGALLYICGTSQMTKRVD
P.berguei        SLLGDMICCKFAIPLTLSGFLLLSLCGANEIFVLLFMTIPLCGGALLYICGTSQMTKRVD
P.yoelii         SLLGDMICCKFAIPLTLSGFLLLSLCGANEFFVLLFMIIPLCGGALLYICGTSQMTKRVD
                 ::***: ***:::****:*** ******** :**:** ********************:

P.vivax          ESELGTAIGLNTSIFYAVTIVAPYLAFKSYLALGLGLYWLLCALICLVITTYIFALDKFT
P.falciparum     ESELGSIIGLNTSLFYAVTIIAPYIAFKSYIALGLGLYWLLCAFICFVVTFYIFVLDKST
P.chabaudi       ESELGSIIGLNTSIFYAVTIMAPYLAFKSYIALGLGLYWLLCAFICFVITIYIFVLDQST
P.berguei        ESELGSIIGLNTSIFYAVTIMAPYLAFKSYIALGLGLYWLLCAFICFVITIYIFVLDQST
P.yoelii         ESELGSIIGLNTSIFYAVTIMAPYLAFKSYIALGLGLYWLLCAFICFVITIYIFVLDQST
                 *****: ******:******:***:*****:***********:**:*:* ***.**: *

P.vivax          LQIFEEDGDSLETMFSSVKLAW
P.falciparum     LKIFKDDKDSIETMFSSIKSIL
P.chabaudi       LKIFEDDADSLETMFSSIKLSL
P.berguei        LKIFENDADSLETMFSSIKLSL
P.yoelii         LQIFENDADSLETMFSSIKLSL
                 *:**::* **:******:*
```

2. *PF11_0825w* mRNA variants

> *PF11_0825w* variant 0

```
    M  E  V  T  S  T  L  L  E  K  G  K  N  F  A  Q  D  P  S  E  V  F  P  E  S
  1 ATGGAAGTAACATCAACCTTATTAGAAAAGGGTAAAAACTTTGCCCAAGATCCATCTGAGGTTTTTCCTGAGTCA

    K  K  F  F  F  S  S  I  G  K  A  H  L  I  N  S  L  Y  G  I  G  Y  T  I  Q
 76 TTTTTTAAAAAAAAAAGCAGTTAACCATTTCGAGTAAACTATTTAAGGGAGATACCTTATCCGATATGATAAGTT

    I  A  M  L  P  Y  L  L  I  S  S  N  A  G  I  E  H  N  G  Y  L  L  T  L  F
151 TAGCGTTACAACGGTATAAACAACTATTCAAGATTACGTCCGTATCTTGTGTTACCTATAAATAATTGGAATAAA

    S  L  L  Q  F  T  G  S  I  F  F  G  R  M  A  D  I  W  G  V  K  K  S  F  Y
226 AGCGAAAATGTTAAATGCCCTAGGTAAAAAAAACCTTCTTACCGTCTGTATACCCCACATTTTTTCAGGAAAATA

    L  S  L  I  S  S  C  L  M  Y  L  M  I  M  V  C  E  S  T  W  A  Y  Y  I  S
301 AATAGAAATTAAAGGAGAACGAATTACATGGATTACTAGTACCACACGCTTAGCTGCACCCGGATGATGTAGTCG

    F  L  P  S  F  F  M  Q  T  F  Q  A  S  S  L  L  V  C  L  K  T  N  F  D  K
376 AAGAATGGTAGGAAAAAATACGTTTGTAAAGTTCGAAGGAGGAATAATCACACGAATTTTTGTTTAAAGCTGTTT

    R  T  G  A  L  G  Y  L  N  L  S  Y  G  M  G  I  I  F  G  S  F  L  A  G  V
451 TCCTGTCCTCGGAATCCAATAGATTTAGATTCAATACCTTACCCATAATATAAGCCATCAAAGAATCGTCCACAA

    M  V  N  F  V  G  S  R  G  N  L  L  I  A  L  L  S  Q  L  I  A  L  C  I  S
526 TACCATTTGAAACATCCTAGTTCTCCTTTAAATAATTAACGTAATAATAGGGTTAATTATCGAAATACATATTCA

    T  T  L  E  E  D  P  K  L  L  K  S  S  N  V  D  K  M  K  M  S  E  I  L  L
601 TGTTGCAATCTTCTTCTAGGCTTTAATAACTTCTCGAGATTACACCTATTTTACTTTTACAGTCTTTATGAAAAT

    S  I  K  N  E  Y  I  R  V  L  N  L  F  K  K  T  Y  G  I  C  L  L  I  L  F
676 TCATAATTTTTACTTATGTATTCTCATAATTTAAATAAATTTTTTTGTATACCTTATACAAATAATTATGAAAAA

    G  L  L  P  I  L  M  T  K  F  A  F  A  P  V  V  V  D  M  F  K  L  T  P  S
751 CCTAATAATGGATATAATTACTGTTTTAAACGAAAACGAGGACACCAACATCTATACAAGTTTAATTGAGGAAGT

    H  T  S  Y  L  M  T  Y  A  G  I  I  T  I  I  A  E  G  I  L  A  P  Y  L  S
826 GTGTGTAGTATAGATTACTGAATACGTCCATATTATTGATAATAACGACTTCCCTATGAACGAGGAATAAATTCA

    S  L  L  G  D  M  I  C  C  K  Y  S  I  P  L  T  L  T  G  F  L  L  L  S  L
901 AGAAATGATCCCCTATACTAAACAACATTTATAAGCTATGGTGATTGTAATTGTCCTAAAAATAATAATAGTAAT

    C  G  A  N  E  S  L  V  L  I  F  M  S  I  P  L  C  G  G  A  L  L  Y  I  C
976 ACACCGCGATTGCTTAGTGAACAAGAATATAAATACAGATATGGTAATACACCTCCACGAAATAATATATATACA

    G  T  S  Q  M  T  K  R  V  E  E  S  E  L  G  S  I  I  G  L  N  T  S  L  F
1051 CCTTGATCGGTTTACTGTTTTGCTCACCTTCTTAGTCTTAACCCAAGCTAATAACCAAATTTATGTAGAGAAAAA

    Y  A  V  T  I  I  A  P  Y  I  A  F  K  S  Y  I  A  L  G  L  G  L  Y  W  L
1126 ATACGGCAATGTTATTATCGAGGTATATAACGGAAATTTAGTATATATCGGAACCCTAACCCTAATATAACCGAG

    L  C  A  F  I  C  F  V  V  T  F  Y  I  F  V  L  D  K  S  T  L  K  I  F  K
1201 GATACACGGAAATAAACAAAACAACAATGAAAGATGTAAAAGCATGAACTATTTAGATGGGAATTTTAAAAATTT

    D  D  K  D  S  I  E  T  M  F  S  S  I  K  S  I  L  *
1276 GACGATAAGGACAGCATAGAAACAATGTTTTCCAGTATTAAGTCGATTTTATAA
```

> *PF11_0825w* variant 1

In green, protein sequence of the longest ORF.

```
      G  S  N  I  N  L  I  R  K  G  *  K  L  C  P  R  S  I  *  G  F  S  *  V  K
    W  K  *  H  Q  P  Y  *  K  R  V  K  T  L  P  K  I  H  L  R  F  F  L  S  Q
    M  E  V  T  S  T  L  L  E  K  G  K  N  F  A  Q  D  P  S  E  V  F  P  E  S
  1 ATGGAAGTAACATCAACCTTATTAGAAAAGGGTAAAAACTTTGCCCAAGATCCATCTGAGGTTTTTCCTGAGTCA

      K  I  F  F  F  V  N  W  *  S  S  F  D  K  F  P  L  W  N  R  L  Y  Y  S  N
    K  N  F  F  F  R  Q  L  V  K  L  I  *  *  I  P  S  M  E  *  A  I  L  F  K
    K  K  F  F  F  S  S  I  G  K  A  H  L  I  N  S  L  Y  G  I  G  Y  T  I  Q
 76 TTTTTTAAAAAAAAAAGCAGTTAACCATTTCGAGTAAACTATTTAAGGGAGATACCTTATCCGATATGATAAGTT

      R  N  V  A  I  F  V  D  K  F  *  C  R  H  R  T  Q  W  I  F  I  N  L  I  F
    S  Q  C  C  H  I  C  *  *  V  L  M  Q  A  *  N  T  M  D  I  Y  *  P  Y  F
    I  A  M  L  P  Y  L  L  I  S  S  N  A  G  I  E  H  N  G  Y  L  L  T  L  F
151 ATCGCAATGTTGCCATATTTGTTGATAAGTTCTAATGCAGGCATAGAACACAATGGATATTTATTAACCTTATTT

      A  F  T  I  Y  G  I  H  F  F  W  K  N  G  R  H  I  M  V  C  E  S  T  W  A
    R  F  Y  N  L  R  D  P  F  F  L  E  E  W  Q  T  Y  H  G  V  R  I  D  V  G
    S  L  L  Q  F  T  G  S  I  F  F  G  R  M  A  D  I  S  W  C  A  N  R  R  G
226 TCGCTTTTACAATTTACGGGATCCATTTTTTTTGGAAGAATGGCAGACATATCATGGTGTGCGAATCGACGTGGG
```

```
     Y  Y  I  S  F  L  P  S  F  F  M  Q  T  F  Q  A  S  S  L  L  V  C  L  K  T
    L  L  H  Q  L  L  T  I  L  F  Y  A  N  I  S  S  F  L  L  I  S  V  L  K  N
    P  T  T  S  A  S  Y  H  P  F  L  C  K  H  F  K  L  P  P  Y  *  C  A  *  K
301 CCTACTACATCAGCTTCTTACCATCCTTTTTTATGCAAACATTTCAAGCTTCCTCCTTATTAGTGTGCTTAAAAA

     N  F  D  K  R  T  G  A  L  G  Y  L  N  L  S  Y  G  M  G  I  I  F  G  S  F
    K  F  R  Q  K  D  R  S  L  R  L  S  K  S  K  L  W  N  G  Y  Y  I  R  *  F
    Q  I  S  T  K  G  Q  E  P  *  V  I  *  I  *  V  M  E  W  V  L  Y  S  V  V
376 CAAATTTCGACAAAAGGACAGGAGCCTTAGGTTATCTAAATCTAAGTTATGGAATGGGTATTATATTCGGTAGTT

     L  A  G  V  M  V  N  F  V  G  S  R  G  N  L  L  I  A  L  L  S  Q  L  I  A
    L  S  R  C  Y  G  K  L  C  R  I  K  R  K  F  I  N  C  I  I  I  P  I  N  S
    S  *  Q  V  L  W  *  T  L  *  D  Q  E  E  I  Y  *  L  H  Y  Y  P  N  *  *
451 TCTTAGCAGGTGTTATGGTAAACTTTGTAGGATCAAGAGGAAATTTATTAATTGCATTATTATCCCAATTAATAG

     L  C  I  S  T  T  L  E  E  D  P  K  L  L  K  S  S  N  V  D  K  M  K  M  S
    F  M  Y  K  Y  N  V  R  R  R  S  E  I  I  E  E  L  *  C  G  *  N  E  N  V
    L  Y  V  *  V  Q  R  *  K  K  I  R  N  Y  *  R  A  L  M  W  I  K  *  K  C
526 CTTTATGTATAAGTACAACGTTAGAAGAAGATCCGAAATTATTGAAGAGCTCTAATGTGGATAAAATGAAAATGT

     E  I  L  L  S  I  K  N  E  Y  I  R  V  L  N  L  F  K  K  T  Y  G  I  C  L
    R  N  T  F  K  Y  *  K  *  I  H  K  S  I  K  F  I  *  K  N  I  W  N  M  F
    Q  K  Y  F  *  V  L  K  M  N  T  *  E  Y  *  I  Y  L  K  K  H  M  E  Y  V
601 CAGAAATACTTTTAAGTATTAAAAATGAATACATAAGAGTATTAAATTTATTTAAAAAAACATATGGAATATGTT

     L  I  L  F  G  L  L  P  I  L  M  T  K  F  A  F  A  P  V  V  V  D  M  F  K
    I  N  T  F  W  I  I  T  Y  I  N  D  K  I  C  F  C  S  C  G  C  R  Y  V  Q
    Y  *  Y  F  L  D  Y  Y  L  Y  *  *  Q  N  L  L  L  L  L  W  L  *  I  C  S
676 TATTAATACTTTTTGGATTATTACCTATATTAATGACAAAATTTGCTTTTGCTCCTGTGGTTGTAGATATGTTCA

     L  T  P  S  H  T  S  Y  L  M  T  Y  A  G  I  I  T  I  I  A  E  G  I  L  A
    I  N  S  F  T  H  I  I  S  N  D  L  C  R  Y  N  N  Y  Y  C  *  R  D  T  C
    N  *  L  L  H  T  H  H  I  *  *  L  M  Q  V  *  *  L  L  L  L  K  G  Y  L
751 AATTAACTCCTTCACACACATCATATCTAATGACTTATGCAGGTATAATAACTATTATTGCTGAAGGGATACTTG

     P  Y  L  S  S  L  L  G  D  M  I  C  C  K  Y  S  I  P  L  T  L  T  G  F  L
    S  L  F  K  F  F  T  R  G  Y  D  L  L  *  I  F  D  T  T  N  I  N  R  I  F
    L  L  I  *  V  L  Y  *  G  I  *  F  V  V  N  I  R  Y  H  *  H  *  Q  D  F
826 CTCCTTATTTAAGTTCTTTACTAGGGGATATGATTTGTTGTAAATATTCGATACCACTAACATTAACAGGATTTT

     L  L  S  L  C  G  A  N  E  S  L  V  L  I  F  M  S  I  P  L  C  G  G  A  L
    I  I  I  I  M  W  R  *  R  I  T  C  S  Y  I  Y  V  Y  T  I  M  W  R  C  F
    Y  Y  Y  H  Y  V  A  L  T  N  H  L  F  L  Y  L  C  L  Y  H  Y  V  E  V  L
901 TATTATTATCATTATGTGGCGCTAACGAATCACTTGTTCTTATATTTATGTCTATACCATTATGTGGAGGTGCTT

     L  Y  I  C  G  T  S  Q  M  T  K  R  V  E  E  S  E  L  G  S  I  I  G  L  N
    I  I  Y  M  W  N  *  P  N  D  K  T  S  G  R  I  R  I  G  F  D  Y  W  F  K
    Y  Y  I  Y  V  E  L  A  K  *  Q  N  E  W  K  N  Q  N  W  V  R  L  L  V  *
976 TATTATATATATGTGGAACTAGCCAAATGACAAAACGAGTGGAAGAATCAGAATTGGGTTCGATTATTGGTTTAA

     T  S  L  F  Y  A  V  T  I  I  A  P  Y  I  A  F  K  S  Y  I  A  L  G  L  G
    Y  I  S  F  L  C  R  Y  N  N  S  S  I  Y  C  L  *  I  I  Y  S  L  G  I  G
    I  H  L  F  F  M  P  L  Q  *  *  L  H  I  L  P  L  N  H  I  *  P  W  D  W
1051 ATACATCTCTTTTTTATGCCGTTACAATAATAGCTCCATATATTGCCTTTAAATCATATATAGCCTTGGGATTGG

     L  Y  W  L  L  C  A  F  I  C  F  V  V  T  F  Y  I  F  V  L  D  K  S  T  L
    I  I  L  A  P  M  C  L  Y  L  F  C  C  Y  F  L  H  F  R  T  *  *  I  Y  P
    D  Y  I  G  S  Y  V  P  L  F  V  L  L  L  L  S  T  F  S  Y  L  I  N  L  P
1126 GATTATATTGGCTCCTATGTGCCTTTATTTGTTTTGTTGTTACTTTCTACATTTTCGTACTTGATAAATCTACCC

     K  I  F  K  D  D  K  D  S  I  E  T  M  F  S  S  I  K  S  I  L  *
    *  N  F  *  R  R  *  G  Q  H  R  N  N  V  F  Q  Y  *  V  D  F  I
    L  K  F  L  K  T  I  R  T  A  *  K  Q  C  F  P  V  L  S  R  F  Y
1201 TTAAAATTTTTAAAGACGATAAGGACAGCATAGAAACAATGTTTTCCAGTATTAAGTCGATTTTATAA
```

> *PF11_0825w* variant 2

In green, protein sequence of the longest ORF; in yellow, protein sequence codified on the 3rd intron that is not present on the other mRNA variants; in lower case, 3rd intron DNA sequence.

```
      G  S  N  I  N  L  I  R  K  G  *  K  L  C  P  R  S  I  *  G  F  S  *  V  K
     W  K  *  H  Q  P  Y  *  K  R  V  K  T  L  P  K  I  H  L  R  F  F  L  S  Q
    M  E  V  T  S  T  L  L  E  K  G  K  N  F  A  Q  D  P  S  E  V  F  P  E  S
  1 ATGGAAGTAACATCAACCTTATTAGAAAAGGGTAAAAACTTTGCCCAAGATCCATCTGAGGTTTTTCCTGAGTCA

      K  I  F  F  F  V  N  W  *  S  S  F  D  K  F  P  L  W  N  R  L  Y  Y  S  N
     K  N  F  F  F  R  Q  L  V  K  L  I  *  *  I  P  S  M  E  *  A  I  L  F  K
    K  K  F  F  F  S  S  I  G  K  A  H  L  I  N  S  L  Y  G  I  G  Y  T  I  Q
 76 AAAAAATTTTTTTTTTCGTCAATTGGTAAAGCTCATTTGATAAATTCCCTCTATGGAATAGGCTATACTATTCAA

      R  N  V  A  I  F  V  D  K  F  *  C  R  H  R  T  Q  W  I  F  I  N  L  I  F
     S  Q  C  C  H  I  C  *  *  V  L  M  Q  A  *  N  T  M  D  I  Y  *  P  Y  F
    I  A  M  L  P  Y  L  L  I  S  S  N  A  G  I  E  H  N  G  Y  L  L  T  L  F
151 ATCGCAATGTTGCCATATTTGTTGATAAGTTCTAATGCAGGCATAGAACACAATGGATATTTATTAACCTTATTT

      A  F  T  I  Y  G  I  H  F  F  W  K  N  G  R  H  V  K  N  I  N  R  I  I  Y
     R  F  Y  N  L  R  D  P  F  F  L  E  E  W  Q  T  C  *  K  Y  K  Q  D  Y  I
    S  L  L  Q  F  T  G  S  I  F  F  G  R  M  A  D  M  L  K  I  *  T  G  L  Y
226 TCGCTTTTACAATTTACGGGATCCATTTTTTTTGGAAGAATGGCAGACATgttaaaaatataaacaggattatat

      Y  R  N  E  R  K  K  K  K  C  L  C  I  Y  I  F  L  F  F  H  I  I  Y  I  D
     L  *  K  *  K  K  E  K  E  M  F  M  Y  L  Y  I  F  I  L  S  Y  N  I  Y  R
    I  I  E  M  K  E  R  K  R  N  V  Y  V  F  I  Y  F  Y  S  F  I  *  Y  I  *
301 attatagaaatgaaagaaagaaaaagaaatgtttatgtatttatatattttattctttcatataatatatatag

      G  V  *  K  S  P  F  I  Y  L  *  F  P  L  A  *  C  T  *  *  S  W  C  A  N
     W  G  V  K  K  S  F  Y  L  S  L  I  S  S  C  L  M  Y  L  M  I  M  V  C  E
    M  G  C  K  K  V  L  L  F  I  F  N  F  L  L  L  N  V  P  N  D  H  G  V  R
376 ATGGGGTGTAAAAAAGTCCTTTTATTTATCTTTAATTTCCTCTTGCTTAATGTACCTAATGATCATGGTGTGCGA

      R  R  G  P  T  T  S  A  S  Y  H  P  F  L  C  K  H  F  K  L  P  P  Y  *  C
     S  T  W  A  Y  Y  I  S  F  L  P  S  F  F  M  Q  T  F  Q  A  S  S  L  L  V
    I  D  V  G  L  L  H  Q  L  L  T  I  L  F  Y  A  N  I  S  S  F  L  L  I  S
451 ATCGACGTGGGCCTACTACATCAGCTTCTTACCATCCTTTTTTATGCAAACATTTCAAGCTTCCTCCTTATTAGT

      A  *  K  Q  I  S  T  K  G  Q  E  P  *  V  I  *  I  *  V  M  E  W  V  L  Y
     C  L  K  T  N  F  D  K  R  T  G  A  L  G  Y  L  N  L  S  Y  G  M  G  I  I
    V  L  K  N  K  F  R  Q  K  D  R  S  L  R  L  S  K  S  K  L  W  N  G  Y  Y
526 GTGCTTAAAAACAAATTTCGACAAAAGGACAGGAGCCTTAGGTTATCTAAATCTAAGTTATGGAATGGGTATTAT

      S  V  V  S  *  Q  V  L  W  *  T  L  *  D  Q  E  E  I  Y  *  L  H  Y  Y  P
     F  G  S  F  L  A  G  V  M  V  N  F  V  G  S  R  G  N  L  L  I  A  L  L  S
    I  R  *  F  L  S  R  C  Y  G  K  L  C  R  I  K  R  K  F  I  N  C  I  I  I
601 ATTCGGTAGTTTCTTAGCAGGTGTTATGGTAAACTTTGTAGGATCAAGAGGAAATTTATTAATTGCATTATTATC

      N  *  *  L  Y  V  *  V  Q  R  *  K  K  I  R  N  Y  *  R  A  L  M  W  I  K
     Q  L  I  A  L  C  I  S  T  T  L  E  E  D  P  K  L  L  K  S  S  N  V  D  K
    P  I  N  S  F  M  Y  K  Y  N  V  R  R  R  S  E  I  I  E  E  L  *  C  G  *
676 CCAATTAATAGCTTTATGTATAAGTACAACGTTAGAAGAAGATCCGAAATTATTGAAGAGCTCTAATGTGGATAA

      *  K  C  Q  K  Y  F  *  V  L  K  M  N  T  *  E  Y  *  I  Y  L  K  K  H  M
     M  K  M  S  E  I  L  L  S  I  K  N  E  Y  I  R  V  L  N  L  F  K  K  T  Y
    N  E  N  V  R  N  T  F  K  Y  *  K  *  I  H  K  S  I  K  F  I  *  K  N  I
751 AATGAAAATGTCAGAAATACTTTTAAGTATTAAAAATGAATACATAAGAGTATTAAATTTATTTAAAAAAACATA

      E  Y  V  Y  *  Y  F  L  D  Y  Y  L  Y  *  *  Q  N  L  L  L  L  W  L  *
     G  I  C  L  L  I  L  F  G  L  L  P  I  L  M  T  K  F  A  F  A  P  V  V  V
    W  N  M  F  I  N  T  F  W  I  I  T  Y  I  N  D  K  I  C  F  C  S  C  G  C
826 TGGAATATGTTTATTAATACTTTTTGGATTATTACCTATATTAATGACAAAATTTGCTTTTGCTCCTGTGGTTGT

      I  C  S  N  *  L  L  H  T  H  H  I  *  *  L  M  Q  V  *  *  L  L  L  L  K
     D  M  F  K  L  T  P  S  H  T  S  Y  L  M  T  Y  A  G  I  I  T  I  I  A  E
    R  Y  V  Q  I  N  S  F  T  H  I  I  S  N  D  L  C  R  Y  N  N  Y  Y  C  *
901 AGATATGTTCAAATTAACTCCTTCACACACATCATATCTAATGACTTATGCAGGTATAATAACTATTATTGCTGA
```

```
       G  Y  L  L  L  I  *  V  L  Y  *  G  I  *  F  V  V  N  I  R  Y  H  *  H  *
      G  I  L  A  P  Y  L  S  S  L  L  G  D  M  I  C  C  K  Y  S  I  P  L  T  L
     R  D  T  C  S  L  F  K  F  F  T  R  G  Y  D  L  L  *  I  F  D  T  T  N  I

 976 AGGGATACTTGCTCCTTATTTAAGTTCTTTACTAGGGGATATGATTTGTTGTAAATATTCGATACCACTAACATT
        Q  D  F  Y  Y  Y  H  Y  V  A  L  T  N  H  L  F  L  Y  L  C  L  Y  H  Y  V
      T  G  F  L  L  L  S  L  C  G  A  N  E  S  L  V  L  I  F  M  S  I  P  L  C
     N  R  I  F  I  I  I  I  M  W  R  *  R  I  T  C  S  Y  I  Y  V  Y  T  I  M
1051 AACAGGATTTTTATTATTATCATTATGTGGCGCTAACGAATCACTTGTTCTTATATTTATGTCTATACCATTATG

       E  V  L  Y  Y  I  Y  V  E  L  A  K  *  Q  N  E  W  K  N  Q  N  W  V  R  L
      G  G  A  L  L  Y  I  C  G  T  S  Q  M  T  K  R  V  E  E  S  E  L  G  S  I
     W  R  C  F  I  I  Y  M  W  N  *  P  N  D  K  T  S  G  R  I  R  I  G  F  D
1126 TGGAGGTGCTTTATTATATATATGTGGAACTAGCCAAATGACAAAACGAGTGGAAGAATCAGAATTGGGTTCGAT

       L  V  *  I  H  L  F  F  M  P  L  Q  *  *  L  H  I  L  P  L  N  H  I  *  P
      I  G  L  N  T  S  L  F  Y  A  V  T  I  I  A  P  Y  I  A  F  K  S  Y  I  A
     Y  W  F  K  Y  I  S  F  L  C  R  Y  N  N  S  S  I  Y  C  L  *  I  I  Y  S
1201 TATTGGTTTAAATACATCTCTTTTTTATGCCGTTACAATAATAGCTCCATATATTGCCTTTAAATCATATATAGC

       W  D  W  D  Y  I  G  S  Y  V  P  L  F  V  L  L  L  L  S  T  F  S  Y  L  I
      L  G  L  G  L  Y  W  L  L  C  A  F  I  C  F  V  V  T  F  Y  I  F  V  L  D
     L  G  I  G  I  I  L  A  P  M  C  L  Y  L  F  C  C  Y  F  L  H  F  R  T  *
1276 CTTGGGATTGGGATTATATTGGCTCCTATGTGCCTTTATTTGTTTTGTTGTTACTTTCTACATTTTCGTACTTGA

       N  L  P  L  K  F  L  K  T  I  R  T  A  *  K  Q  C  F  P  V  L  S  R  F  Y
      K  S  T  L  K  I  F  K  D  D  K  D  S  I  E  T  M  F  S  S  I  K  S  I  L
     *  I  Y  P  *  N  F  *  R  R  *  G  Q  H  R  N  N  V  F  Q  Y  *  V  D  F
1351 TAAATCTACCCTTAAAATTTTTAAAGACGATAAGGACAGCATAGAAACAATGTTTTCCAGTATTAAGTCGATTTT

      *
     I
1426 ATAA
```

3. Alignment N-terminal PFE0825w variants

```
variant0      MEVTSTLLEKGKNFAQDPSEVFPESKKFFFSSIGKAHLINSLYGIGYTIQIAMLPYLLIS
variant1      ------------------------------------------------------------
variant2      ------------------------------------------------------------

variant0      SNAGIEHNGYLLTLFSLLQFTGSIFFGRMADIWGVKKSFYLSLISSCLMYLMIMVCESTW
variant1      ----------------------------------------------------MVCESTW
variant2      ----------------MFMYLYIFILSYNIYRWGVKKSFYLSLISSCLMYLMIMVCESTW
                                                                 *******
```

4. Codon optimized sequences for expression in *X. laevis*

> *PF11_0825w* variant 0

```
   1 ATGGAAGTTA CTTCTACCTT GTTGGAAAAG GGTAAGAACT TTGCTCAAGA TCCATCTGAA
  61 GCTGCTCCAG AATCTAAAAA AGCTGCTTTC TCATCCATTG GTAAGGCCCA TTTGATCAAT
 121 TCCTTGTATG GTATTGGTTA CACCATCCAA ATTGCCATGT TGCCATACTT GTTGATTTCT
 181 TCTAACGCCG GTATCGAACA TAACGGTTAT TTGTTGACCT TGTTCTCCTT GTTGCAATTC
 241 ACCGGTTCTA TTTTCTTCGG TAGAATGGCT GATATCTGGG GTGTTAAGAA GTCTTTCTAC
 301 TTGTCTTTGA TCTCCTCCTG CTTGATGTAC TTGATGATTA TGGTTTGTGA ATCCACCTGG
 361 GCTTACTACA TTTCTTTTTT GCCATCCTTC TTCATGCAAA CCTTCCAAGC TTCTTCTTTG
 421 TTGGTCTGCT TAAAGACCAA CTTCGATAAG AGAACTGGTG CTTTGGGTTA CTTGAATTTG
 481 TCTTATGGTA TGGGTATCAT CTTCGGTTCT TTTTTGGCTG GTGTTATGGT TAACTTCGTT
 541 GGTTCTAGAG GTAACTTGTT GATTGCTTTG TTGTCCCAAT TGATTGCCTT GTGTATTTCT
 601 ACCACCTTGG AAGAAGATCC AAAGTTGTTG AAGTCCTCCA ACGTTGATAA GATGAAGATG
 661 TCCGAAATCT TGTTGTCCAT CAAGAACGAG TATATCAGAG TCTTGAACTT GTTCAAAAAG
 721 ACCTACGGTA TCTGCTTGTT GATCTTGTTT GGTTTGTTGC CAATCTTGAT GACCAAGTTT
 781 GCTTTTGCTC CAGTTGTTGT TGACATGTTT AAGTTGACTC CATCCCATAC CTCTTACTTG
 841 ATGACATACG CTGGTATCAT TACCATTATT GCCGAAGGTA TTTTGGCCCC ATACTTGTCA
 901 TCTTTGTTGG GTGATATGAT CTGTTGCAAG TACTCTATTC CATTGACTTT GACCGGTTTC
 961 TTGTTGTTGT CTTTGTGTGG TGCTAACGAA TCCTTGGTTT TGATTTTCAT GTCCATTCCA
1021 TTGTGTGGTG GTGCTTTGTT GTACATTTGT GGTACTTCTC AAATGACCAA GAGAGTTGAA
```

```
1081 GAATCTGAAT TGGGTTCCAT TATCGGTTTG AACACCTCTT TGTTCTACGC CGTTACTATT
1141 ATTGCTCCTT ACATTGCTTT CAAGTCCTAC ATTGCTTTGG GTTTGGGTCT ATATTGGTTG
1201 TTGTGTGCTT TCATCTGCTT CGTTGTTACC TTCTACATCT TCGTTTTGGA TAAGTCCACC
1261 TTGAAGATTT TCAAAGCTGC TAAGGCTTCC ATTGCTACTA TGTTCTCTTC CATCAAATCC
1321 ATCTTGCACC ACCACCACCA CCACTAA
```

> *PF11_0825w* variant 1

```
   1 ATGGTTTGTG AATCCACCTG GGCTTACTAC ATTTCTTTTT TGCCATCCTT CTTCATGCAA
  61 ACCTTCCAAG CTTCTTCTTT GTTGGTCTGC TTAAAGACCA ACTTCGATAA GAGAACTGGT
 121 GCTTTGGGTT ACTTGAATTT GTCTTATGGT ATGGGTATCA TCTTCGGTTC TTTTTTGGCT
 181 GGTGTTATGG TTAACTTCGT TGGTTCTAGA GGTAACTTGT TGATTGCTTT GTTGTCCCAA
 241 TTGATTGCCT TGTGTATTTC TACCACCTTG GAAGAAGATC CAAAGTTGTT GAAGTCCTCC
 301 AACGTTGATA AGATGAAGAT GTCCGAAATC TTGTTGTCCA TCAAGAACGA GTATATCAGA
 361 GTCTTGAACT TGTTCAAAAA GACCTACGGT ATCTGCTTGT TGATCTTGTT TGGTTTGTTG
 421 CCAATCTTGA TGACCAAGTT TGCTTTTGCT CCAGTTGTTG TTGACATGTT TAAGTTGACT
 481 CCATCCCATA CCTCTTACTT GATGACATAC GCTGGTATCA TTACCATTAT TGCCGAAGGT
 541 ATTTTGGCCC CATACTTGTC ATCTTTGTTG GGTGATATGA TCTGTTGCAA GTACTCTATT
 601 CCATTGACTT TGACCGGTTT CTTGTTGTTG TCTTTGTGTG GTGCTAACGA ATCCTTGGTT
 661 TTGATTTTCA TGTCCATTCC ATTGTGTGGT GGTGCTTTGT TGTACATTTG TGGTACTTCT
 721 CAAATGACCA AGAGAGTTGA AGAATCTGAA TTGGGTTCCA TTATCGGTTT GAACACCTCT
 781 TTGTTCTACG CCGTTACTAT TATTGCTCCT TACATTGCTT TCAAGTCCTA CATTGCTTTG
 841 GGTTTGGGTC TATATTGGTT GTTGTGTGCT TTCATCTGCT TCGTTGTTAC CTTCTACATC
 901 TTCGTTTTGG ATAAGTCCAC CTTGAAGATT TTCAAAGCTG CTAAGGCTTC CATTGCTACT
 961 ATGTTCTCTT CCATCAAATC CATCTTGCAC CACCACCACC ACCACTAA
```

> *PF11_0825w* variant 2

In yellow, protein sequence codified on the 3rd intron that is not present on the other mRNA variants and that was not codon optimized.

```
   1 ATGTTTATGT ATTTATATAT TTTTATTCTT TCATATAATA TATATAGATG GGGTGTAAAA
  61 AAGTCCTTTT ATTTATCTTT AATTTCCTCT TGCTTAATGT ACCTAATGAT CATGGTTTGT
 121 GAATCCACCT GGGCTTACTA CATTTCTTTT TTGCCATCCT TCTTCATGCA AACCTTCCAA
 181 GCTTCTTCTT TGTTGGTCTG CTTAAAGACC AACTTCGATA AGAGAACTGG TGCTTTGGGT
 241 TACTTGAATT TGTCTTATGG TATGGGTATC ATCTTCGGTT CTTTTTTGGC TGGTGTTATG
 301 GTTAACTTCG TTGGTTCTAG AGGTAACTTG TTGATTGCTT TGTTGTCCCA ATTGATTGCC
 361 TTGTGTATTT CTACCACCTT GGAAGAAGAT CCAAAGTTGT TGAAGTCCTC CAACGTTGAT
 421 AAGATGAAGA TGTCCGAAAT CTTGTTGTCC ATCAAGAACG AGTATATCAG AGTCTTGAAC
 481 TTGTTCAAAA AGACCTACGG TATCTGCTTG TTGATCTTGT TTGGTTTGTT GCCAATCTTG
 541 ATGACCAAGT TTGCTTTTGC TCCAGTTGTT GTTGACATGT TTAAGTTGAC TCCATCCCAT
 601 ACCTCTTACT TGATGACATA CGCTGGTATC ATTACCATTA TTGCCGAAGG TATTTTGGCC
 661 CCATACTTGT CATCTTTGTT GGGTGATATG ATCTGTTGCA AGTACTCTAT TCCATTGACT
 721 TTGACCGGTT TCTTGTTGTT GTCTTTGTGT GGTGCTAACG AATCCTTGGT TTTGATTTTC
 781 ATGTCCATTC CATTGTGTGG TGGTGCTTTG TTGTACATTT GTGGTACTTC TCAAATGACC
 841 AAGAGAGTTG AAGAATCTGA ATTGGGTTCC ATTATCGGTT TGAACACCTC TTTGTTCTAC
 901 GCCGTTACTA TTATTGCTCC TTACATTGCT TTCAAGTCCT ACATTGCTTT GGGTTTGGGT
 961 CTATATTGGT TGTTGTGTGC TTTCATCTGC TTCGTTGTTA CCTTCTACAT CTTCGTTTTG
1021 GATAAGTCCA CCTTGAAGAT TTTCAAAGCT GCTAAGGCTT CCATTGCTAC TATGTTCTCT
1081 TCCATCAAAT CCATCTTGCA CCACCACCAC CACCACTAA
```

> *PfCRT*^{Dd2}

```
   1 ATGAAGTTCG CCTCTAAGAA GAACAATCAA AAGAACTCCT CCAAGAATGC TGAAAGAGCT
  61 AGAGCTGCTG ATAATGCTGC TCAAGAAGGT AACGGTTCTA GATTGGGTGG TGGTTCTTGT
 121 TTGGGTAAAT GTGCTCATGC TGCTAAAGCT GCCTTCAAAG AAATCAAGGA CAACATCTTC
 181 ATCTACATCT TGTCCATCAT CTACTTGTCC GTTTGCGTTA TTGAAACCAT CTTCGCCAAG
 241 AGAACCTTGA ACAAGATTGG TAACTACTCT TTCGTTACCT CTGAAACCCA TAACTTCATC
 301 TGCATGATCA TGTTCTTCAT CGTCTATTCC TTGTTCGGTA ACAAGAAGGG TAACTCCAAA
 361 GAAAGACACA GATCCTTCAA CTTGCAATTC TTCGCCATTT CTATGTTGGA TGCCTGCTCT
 421 GTTATTTTGG CTTTCATCGG TTTGACTAGA ACTACCGGTA ACATCCAATC TTTCGTCTTG
 481 CAATTGTCCA TTCCAATCAA TATGTTCTTC TGCTTCTTGA TCTTGAGATA CAGATACCAC
 541 TTGTACAATT ACTTGGGTGC CGTTATTATT GTCGTTACCA TTGCCTTGGT TGAAATGAAG
 601 TTGTCCTTCG AAACCCAAGA AGAAAACTCC ATCATCTTCA ACTTGGTTTT GATCTCCTCA
 661 TTGATCCCAG TTTGTTTCTC TAACATGACC AGAGAAATCG TTTTCAAGAA GTACAAGATC
 721 GACATCTTGA GATTGAACGC TATGGTTTCC TTCTTCCAAT TATTCACCTC CTGCTTGATT
 781 TTGCCAGTTT ACACCTTGCC ATTCTTGAAA GAATTGCACT TGCCATACAA CGAAATTTGG
 841 ACCAACATCA AGAATGGTTT CGCTTGTTTG TTCTTGGGTA GAAACACCGT TGTTGAAAAC
 901 TGTGGTTTGG GTATGGCTAA GTTGTGTGAT GATTGTGATG GTGCTTGGAA AACTTTCGCT
 961 TTGTTCTCCT TCTTCTCCAT TTGCGATAAC TTGATCACCT CCTACATTAT CGATAAGTTC
1021 TCCACTATGA CCTACACTAT CGTATCTTGC ATTCAAGGTC CAGCTACTGC TATTGCTTAC
1081 TACTTCAAGT TCTTGGCTGG TGATGTTGTT ATTGAACCTA GATTATTGGA CTTCGTCACC
1141 TTGTTTGGTT ACTTGTTCGG TTCCATTATC TACAGAGTCG GTAACATCAT CTTGGAAAGA
1201 AAGAAGATGA GAAACGAAGA AAACGCTGAT TCTGCTGGTG CTTTGACTAA TGTTGATTCT
1261 GCTGCTACTC AACCTAGGTA A
```

5. Mutagenesis of PfCRT^{S33A}

> *PfCRT* homology region

In green, *pfcrt* ATG starting codon; in red, S^{33}A mutation; in lower case, non coding DNA sequences.

```
   1 aaatatttta aaatcgacat tccgatatat tatattttta gactataata tccgttaata
  61 ataaatacac gcagtcatat tatttattat acattcattt attattttgt ttttttaat
 121 ttcttacata taacaaaATG AAATTCGCAA GTAAAAAAAA TAATCAAAAA AATTCAAGCA
 181 AAAATGACGA GCGTTATAGA GAATTAGATA ATTTAGTACA AGAAGGAAgt aagtatccaa
 241 aaatggaaat attgaatgat ataaatgaat agataaatca acctattgga tatatatata
 301 tatatatata tatatatata tatgtatacc catatgtatt aatttttttt ttttttttt
 361 ttttttttt ttttttttt cccttgtcga ccttaacagA TGGAGCACGT TTAGGTGGAG
 421 GTTCTTGTCT TGGTAAATGT GCTCATGTGT TTAAACTTAT TTTTAAAGAG ATTAAGGATA
 481 ATATTTTTAT TTATATTTTA AGTATTATTT ATTTAAGTGT ATGTGTAATT GAAACAATTT
 541 TTGCTAAAAG AACTTTAAAC AAAATTGGTA ACTATAGTTT TGTAACATCC GAAACTCACA
 601 ACTTTATTTG TATGATTATG TTCTTTATTG TTTATTCCTT ATTTGGAAAT AAAAAGGGAA
 661 ATTCAAAAgt aagataaatc aatatattaa aatgatggat ttataagaga atctattcca
 721 cctaccaata taaaacatta cacatatata tatatatata tatatatata tatgtatgta
 781 tgttgattaa tttgtttata tatttatatt tatttcttat gacctttta gGAACGACAC
 841 CGAAGCTTTA ATTTACAATT TTTTGCTATA TCCATGTTAG ATGCCTGTTC AGT
```

> *PfCRT guide 3*

```
   1 TAAACGTGAG CCATCTGTTA
```

6. Episomal PfCRT^{Dd2}-GFP overexpression

> *PfCRT*^{Dd2}*-GFP*

In green, GFP tag coding sequence.

```
   1 ATGAAATTCG CAAGTAAAAA AAATAATCAA AAAAATTCAA GCAAAAATGA CGAGCGTTAT
  61 AGAGAATTAG ATAATTTAGT ACAAGAAGGA AATGGCTCAC GTTTAGGTGG AGGTTCTTGT
 121 CTTGGTAAAT GTGCTCATGT GTTTAAACTT ATTTTTAAAG AGATTAAGGA TAACATTTTT
 181 ATTTATATTT TAAGTATTAT TTATTTAAGT GTATGTGTAA TTGAAACAAT TTTTGCTAAA
 241 AGAACCTTAA ACAAAATTGG TAACTATAGT TTTGTAACAT CCGAAACTCA CAACTTTATT
 301 TGTATGATTA TGTTCTTTAT TGTTTATTCC TTATTTGGAA ATAAAAAGGG AAATTCAAAA
```

```
 361 GAACGACACC GAAGCTTTAA TTTACAATTT TTTGCTATAT CCATGTTAGA TGCCTGTTCA
 421 GTCATTTTGG CCTTCATAGG TCTTACAAGA ACTACTGGAA ATATCCAATC ATTTGTTCTT
 481 CAATTAAGTA TTCCTATTAA TATGTTCTTC TGCTTTTTAA TATTAAGGTA TAGATATCAC
 541 TTATACAATT ATCTCGGAGC AGTTATTATT GTTGTAACAA TAGCTCTTGT AGAAATGAAA
 601 TTATCTTTTG AAACACAAGA AGAAAATTCT ATCATATTTA ATCTTGTCTT AATTAGTTCC
 661 TTAATTCCTG TATGCTTTTC AAACATGACA AGGGAAATAG TTTTTAAAAA ATATAAGATT
 721 GACATTTTAA GATTAAATGC TATGGTATCC TTTTTCCAAT TGTTCACTTC TTGTCTTATA
 781 TTACCTGTAT ACACCCTTCC ATTTTTAAAA GAACTTCATT TACCATATAA TGAAATATGG
 841 ACAAATATAA AAAATGGTTT CGCATGTTTA TTCTTGGGAA GAAACACAGT CGTAGAGAAT
 901 TGTGGTCTTG GTATGGCTAA GTTATGTGAT GATTGTGACG GAGCATGGAA AACCTTCGCA
 961 TTGTTTTCCT TCTTTAGCAT TTGTGATAAT TTAATAACCA GCTATATTAT CGACAAATTT
1021 TCTACCATGA CATATACTAT TGTTAGTTGT ATACAAGGTC CAGCAACAGC AATTGCTTAT
1081 TACTTTAAAT TCTTAGCCGG TGATGTTGTA ATAGAACCAA GATTATTAGA TTTCGTAACT
1141 TTGTTTGGCT ACCTATTTGG TTCTATAATT TACCGTGTAG GAAATATTAT CTTAGAAAGA
1201 AAAAAAATGA GAAATGAAGA AAATGAAGAT TCCGAAGGAG AATTAACCAA CGTCGATTCA
1261 ATTATTACAC AATAACCTAG GAGTAAAGGA GAAGAACTTT TCACTGGAGT TGTCCCAATT
1321 CTTGTTGAAT TAGATGGTGA TGTTAATGGG CACAAATTTT CTGTCAGTGG AGAGGGTGAA
1381 GGTGATGCAA CATACGGAAA ACTTACCCTT AAATTTATTT GCACTACTGG AAAACTACCT
1441 GTTCCATGGC CAACACTTGT CACTACTTTC GCGTATGGTC TTCAATGCTT TGCGAGATAC
1501 CCAGATCATA TGAAACAGCA TGACTTTTTC AAGAGTGCCA TGCCCGAAGG TTATGTACAG
1561 GAAAGAACTA TATTTTTCAA AGATGACGGG AACTACAAGA CACGTGCTGA AGTCAAGTTT
1621 GAAGGTGATA CCCTTGTTAA TAGAATCGAG TTAAAAGGTA TTGATTTTAA AGAAGATGGA
1681 AACATTCTTG GACACAAATT GGAATACAAC TATAACTCAC ACAATGTATA CATCATGGCA
1741 GACAAACAAA AGAATGGAAT CAAAGTTAAC TTCAAAATTA GACACAACAT TGAAGATGGA
1801 AGCGTTCAAC TAGCAGACCA TTATCAACAA AATACTCCAA TTGGCGATGG CCCTGTCCTT
1861 TTACCAGACA ACCATTACCT GTCCACACAA TCTGCCCTTT CGAAAGATCC CAACGAAAAG
1921 AGAGACCACA TGGTCCTTCT TGAGTTTGTA ACAGCTGCTG GGATTACACA TGGCATGGAT
1981 GAACTATACA AATAA
```

7. PfCK2α-GST and PfCK2α^{K72M}-GST expression

> PfCK2α-GST

In grey, GST tag coding sequence; in pink, thrombin cleavage site.

```
   1 ATGTCCCCTA TACTAGGTTA TTGGAAAATT AAGGGCCTTG TGCAACCCAC TCGACTTCTT
  61 TTGGAATATC TTGAAGAAAA ATATGAAGAG CATTTGTATG AGCGCGATGA AGGTGATAAA
 121 TGGCGAAACA AAAAGTTTGA ATTGGGTTTG GAGTTTCCCA ATCTTCCTTA TTATATTGAT
 181 GGTGATGTTA AATTAACACA GTCTATGGCC ATCATACGTT ATATAGCTGA CAAGCACAAC
 241 ATGTTGGGTG GTTGTCCAAA AGAGCGTGCA GAGATTTCAA TGCTTGAAGG AGCGGTTTTG
 301 GATATTAGAT ACGGTGTTTC GAGAATTGCA TATAGTAAAG ACTTTGAAAC TCTCAAAGTT
 361 GATTTTCTTA GCAAGCTACC TGAAATGCTG AAAATGTTCG AAGATCGTTT ATGTCATAAA
 421 ACATATTTAA ATGGTGATCA TGTAACCCAT CCTGACTTCA TGTTGTATGA CGCTCTTGAT
 481 GTTGTTTTAT ACATGGACCC AATGTGCCTG GATGCGTTCC CAAAATTAGT TTGTTTTAAA
 541 AAACGTATTG AAGCTATCCC ACAAATTGAT AAGTACTTGA AATCCAGCAA GTATATAGCA
 601 TGGCCTTTGC AGGGCTGGCA AGCCACGTTT GGTGGTGGCG ACCATCCTCC AAAATCGGAT
 661 CTGGTTCCGC GTGGATCCAT GTCGGTTAGC TCAATTAATA AAAAAATTTA TATACCAAAA
 721 TTTTATGCTG ATGTCAATAT TCATAAGCCT AAAGAATACT ATGATTATGA TAATTTAGAA
 781 TTACAATGGA ATAAACCAAA TCGTTATGAG ATTATGAAAA AGATTGGGAG GGGAAAATAC
 841 AGTGAGGTGT TTAATGGATA TGATACGGAA TGTAATAGAC CATGTGCTAT TAAAGTATTA
 901 AAGCCTGTTA AAAAAAAAAA AATAAAAAGA GAAATAAAAA TTTTACAAAA TTTGAATGGT
 961 GGTCCAAATA TAATAAAACT ATTAGATATA GTTAAAGATC CTGTTACGAA AACACCATCT
1021 TTAATATTTG AATATATTAA CAATATAGAT TTTAAAACAT TATATCCTAA ATTTACAGAT
1081 AAGGATATTC GTTATTATAT CTATCAAATT TTAAAAGCAT TGGATTATTG TCATAGCCAA
1141 GGTATTATGC ATAGAGATGT TAAACCACAT AATATTATGA TTGATCATGA AAATAGACAA
1201 ATTAGATTAA TTGATTGGGG TCTAGCTGAA TTTTATCATC CTGGTCAAGA ATATAATGTT
1261 CGTGTAGCAA GTAGATATTA TAAAGGTCCA GAACTTTTGA TCGATTTACA ACTTTATGAT
1321 TATTCATTAG ATATATGGAG CCTAGGTTGT ATGCTTGCTG GTATGATCTT TAAAAAGGAA
1381 CCTTTCTTTT GTGGTCATGA TAATTATGAT CAATTAGTTA AAATTGCAAA AGTTCTAGGA
1441 ACAGAAGATC TACATGCTTA CCTAAAAAAA TATAACATTA AACTTAAACC ACATTATCTT
1501 AATATCTTAG GAGAATATGA AAGAAAACCA TGGTCCCATT TTTTAACCCA ATCAAATATT
1561 GATATAGCAA AAGATGAAGT AATTGATCTA ATCGACAAAA TGTTGATTTA TGATCACGCA
1621 AAAAGAATCG CACCAAAGGA AGCCATGGAG CATCCTTACT TTAGAGAAGT CCGTGAGGAA
1681 TCATAA
```

> PfCK2α K72M mutation

```
214 AAA to ATG
     K      M
```

8. Episomal PfCRT^{Dd2}-HA overexpression

> *PfCRT^{Dd2}-HA*

In blue, 3xHA tag coding sequence.

```
   1 ATGAAATTCG CAAGTAAAAA AAATAATCAA AAAAATTCAA GCAAAAATGA CGAGCGTTAT
  61 AGAGAATTAG ATAATTTAGT ACAAGAAGGA AATGGCTCAC GTTTAGGTGG AGGTTCTTGT
 121 CTTGGTAAAT GTGCTCATGT GTTTAAACTT ATTTTTAAAG AGATTAAGGA TAATATTTTT
 181 ATTTATATTT TAAGTATTAT TTATTTAAGT GTATGTGTAA TTGAAACAAT TTTTGCTAAA
 241 AGAACTTTAA ACAAAATTGG TAACTATAGT TTTGTAACAT CCGAAACTCA CAACTTTATT
 301 TGTATGATTA TGTTCTTTAT TGTTTATTCC TTATTTGGAA ATAAAAAGGG AAATTCAAAA
 361 GAACGACACC GAAGCTTTAA TTTACAATTT TTTGCTATAT CCATGTTAGA TGCCTGTTCA
 421 GTCATTTTGG CCTTCATAGG TCTTACAAGA ACTACTGGAA ATATCCAATC ATTTGTTCTT
 481 CAATTAAGTA TTCCTATTAA TATGTTCTTC TGCTTTTTAA TATTAAGATA TAGATATCAC
 541 TTATACAATT ATCTCGGAGC AGTTATTATT GTTGTAACAA TAGCTCTTGT AGAAATGAAA
 601 TTATCTTTTG AAACACAAGA AGAAAATTCT ATCATATTTA ATCTTGTCTT AATTAGTTCC
 661 TTAATTCCTG TATGCTTTTC AAACATGACA AGGGAAATAG TTTTTAAAAA ATATAAGATT
 721 GACATTTTAA GATTAAATGC TATGGTATCC TTTTTCCAAT TGTTCACTTC TTGTCTTATA
 781 TTACCTGTAT ACACCCTTCC ATTTTTAAAA GAACTTCATT TACCATATAA TGAAATATGG
 841 ACAAATATAA AAAATGGTTT CGCATGTTTA TTCTTGGGAA GAAACACAGT CGTAGAGAAT
 901 TGTGGTCTTG GTATGGCTAA GTTATGTGAT GATTGTGACG GAGCATGGAA AACCTTCGCA
 961 TTGTTTTCCT TCTTTAGCAT TTGTGATAAT TTAATAACCA GCTATATTAT CGACAAATTT
1021 TCTACCATGA CATATACTAT TGTTAGTTGT ATACAAGGTC CAGCAACAGC AATTGCTTAT
1081 TACTTTAAAT TCTTAGCCGG TGATGTTGTA ATAGAACCAA GATTATTAGA TTTCGTAACT
1141 TTGTTTGGCT ACCTATTTGG TTCTATAATT TACCGTGTAG GAAATATTAT CTTAGAAAGA
1201 AAAAAAATGA GAAATGAAGA AAATGAAGAT TCCGAAGGAG AATTAACCAA CGTCGATTCA
1261 ATTATTACAC AATAACCTAG GGGCGGTGGA TACCCTTACG ATGTGCCTGA TTACGCGTAT
1321 CCCTATGACG TACCAGACTA TGCATACCCT TATGACGTTC CGGATTATGC TCACGGGGTG
1381 TAA
```

9. Y2H bait sequences

> *PfCRT^{Dd2} N-terminal*

```
   1 ATGAAGTTCG CTTCTAAGAA GAACAACCAA AAGAACTCTT CTAAGAACGA CGAAAGATAC
  61 AGAGAATTGG ACAACTTGGT TCAAGAAGGT AACGGTTCTA GATTGGGTGG TGGTTCTTGT
 121 TTGGGTAAGT GTGCTCACGT TTTCAAGTTG ATTTTCAAGG AAATTAAGGA CAACTAA
```

> *PfCRT^{Dd2} putative calmodulin binding site*

```
   1 TCTGTTTGTG TTATGAACAA GATTTTCGCT AAGAGAACTT TGAACAAGAT TGGTAACTAC
  61 TCTTAA
```

> *PfCRT^{Dd2} out loop*

```
   1 ACTTTGCCAT TCTTGAAGGA ATTGCACTTG CCATACAACG AAATTTGGAC TAACATTAAG
  61 AACGGTTTCG CTTGTTTGTT CTTGGGTAGA AACACTGTTG TTGAAAACTG TGGTTTGGGT
 121 ATGGCTAAGT TGTGTGACGA CTGTGACGGT TAA
```

> *PfCRT^{Dd2} C-terminal*

```
   1 GAAAGAAAGA AGATGAGAAA CGAAGAAAAC GAAGACTCTG AAGGTGAATT GACTAACGTT
  61 GACTCTATTA TTACTCAA
```

10. PF11_0488 sequences

> *PF11_0488 $^{C\text{-}terminal}$-GFP-CAD*

In green, GFP tag coding sequence; in blue 4xCAD tag coding sequence.

```
   1 ATGAAATTAA ATTTGGATAA AAAAAGAGCA ATACTTGAAA AACGATTAGA TCATTTTAAT
  61 TTCCAAGAAA ACTCAGAATT CTCATTTTAT AATCCATTAA AAATAAATAT AAGAATGATG
 121 AACTTAATTG GAAGAGGAGG ATTTGCTGAA GTGTGGGAAG TTTTTGATTC TATCAATTTA
 181 GAAATGTATG CAGCCAAAAT TCATAAAATT GAACCAAGTA TGTCCAATGA AATAAAAAAT
 241 AAAATTATTC AAAGAGCAGA AAATGAAATA AATATACATA TACATTGTCA TAGACATATA
 301 TTTATTGTTA AATTAGAATT CTTTTTTGTA TTTGGTTCAG CAACAAATTT ATTAGTTGGA
 361 ATGGAATTAT GTGATATTGA TCTAGATAAA TATATTAAAT ATCATGGGCC AATTAATGAA
 421 CTTTTAGCTT TATGTTGGAT TAAACAAATA TTATTAGGCT TATTATATAT GAAAAATTTA
 481 CCAACTGGAA AAGTACACCA TTGTGATTTA AAACCTGCCA ACTTATTAAT CAAGGATGGA
 541 ATTATAAAAA TATCCGACTT TGGACTAGCC AAACTAATTT TACCAGATAC ACATCAATAT
 601 TACAATGGAG GTGGTACATT GTATTATCAA CCACCAGAAT GTTTAAAAAA TAAAAAAAAC
 661 CTTCTTATCA CAGATAAAAT TGATATCTGG TCATTGGGAT GCATTCTTTA TGAAATGCTC
 721 TTTTGTGAAA GACCTTTCCA ATTTAATTAC CTTGAAAAAT GTTCAAAAGA ATTATTAGTT
 781 AACAAAATGA AAAATGGATT AACCTATCCA AAAATTAATC AAAAAATTTC TAATGCTACT
 841 TTAAGTTACA TACAATATTT ACTAAATTTT GACTATGAAT TACGACCATC TATAGAAGAA
 901 GCCTTAAGCT ATCCAATTTT TAACTACTTT AATATACCAC CTAGGAGTAA AGGAGAAGAA
 961 CTTTTCACTG GAGTTGTCCC AATTCTTGTT GAATTAGATG GTGATGTTAA TGGGCACAAA
1021 TTTTCTGTCA GTGGAGAGGG TGAAGGTGAT GCAACATACG GAAAACTTAC CCTTAAATTT
1081 ATTTGCACTA CTGGAAAACT ACCTGTTCCA TGGCCAACAC TTGTCACTAC TTTCGCGTAT
1141 GGTCTTCAAT GCTTTGCGAG ATACCCAGAT CATATGAAAC AGCATGACTT TTTCAAGAGT
1201 GCCATGCCCG AAGGTTATGT ACAGGAAAGA ACTATATTTT TCAAAGATGA CGGGAACTAC
1261 AAGACACGTG CTGAAGTCAA GTTTGAAGGT GATACCCTTG TTAATAGAAT CGAGTTAAAA
1321 GGTATTGATT TTAAAGAAGA TGGAAACATT CTTGGACACA AATTGGAATA CAACTATAAC
1381 TCACACAATG TATACATCAT GGCAGACAAA CAAAAGAATG GAATCAAAGT TAACTTCAAA
1441 ATTAGACACA ACATTGAAGA TGGAAGCGTT CAACTAGCAG ACCATTATCA ACAAAATACT
1501 CCAATTGGCG ATGGCCCTGT CCTTTTACCA GACAACCATT ACCTGTCCAC ACAATCTGCC
1561 CTTTCGAAAG ATCCCAACGA AAAGAGAGAC CACATGGTCC TTCTTGAGTT TGTAACAGCT
1621 GCTGGGATTA CACATGGCAT GGATGAACTA TACAAAGGTA CCGGAGTGCA GGTGGAAACC
1681 ATCTCCCCGG GAGACGGGCG CACCTTCCCC AAGCGCGGCC AGACCTGCGT GGTGCACTAC
1741 ACCGGGATGC TTGAAGATGG AAAGAAAATG GATTCCTCCC GGGACAGAAA CAAGCCCTTT
1801 AAGTTTATGC TAGGCAAGCA GGAGGTGATC CGAGGCTGGG AAGAAGGGGT TGCCCAGATG
1861 AGTGTGGGTC AGAGAGCCAA ACTGACTATA TCTCCAGATT ATGCCTATGG TGCCACTGGG
1921 CACCCAGGCA TCATCCCACC ACATGCCACT CTCGTCTTCG ATGTGGAGCT TCTAAAACTG
1981 GAAGTCGAGG GCGTGCAGGT GGAAACCATC TCCCCAGGAG ACGGGCGCAC CTTCCCCAAG
2041 CGCGGCCAGA CCTGCGTGGT GCACTACACC GGGATGCTTG AAGATGGAAA GAAAATGGAT
2101 TCCTCCCGGG ACAGAAACAA GCCCTTTAAG TTTATGCTAG GCAAGCAGGA GGTGATCCGA
2161 GGCTGGGAAG AAGGGGTTGC CCAGATGAGT GTGGGTCAGA GAGCCAAACT GACTATATCT
2221 CCAGATTATG CCTATGGTGC CACTGGGCAC CCAGGCATCA TCCCACCACA TGCCACTCTC
2281 GTCTTCGATG TGGAGCTTCT AAAACTGGAA ACTAGAGGAG TGCAGGTGGA AACCATCTCC
2341 CCAGGAGACG GGCGCACCTT CCCCAAGCGC GGCCAGACCT GCGTGGTGCA CTACACCGGG
2401 ATGCTTGAAG ATGGAAAGAA AATGGATTCC TCCCGGGACA GAAACAAGCC CTTTAAGTTT
2461 ATGCTAGGCA AGCAGGAGGT GATCCGAGGC TGGGAAGAAG GGGTTGCCCA GATGAGTGTG
2521 GGTCAGAGAG CCAAACTGAC TATATCTCCA GATTATGCCT ATGGTGCCAC TGGGCACCCA
2581 GGCATCATCC CACCACATGC CACTCTCGTC TTCGATGTGG AGCTTCTAAA ACTGGAAACT
2641 AGAGGAGTGC AGGTGGAAAC CATCTCCCCG GGAGACGGGC GCACCTTCCC CAAGCGCGGC
2701 CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAAAT GGATTCCTCC
2761 CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG
2821 GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT
2881 TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC
2941 GATGTGGAGC TTCTAAAACT GGAAGGTACC CCGGGTCGAG GGATATGGCA GCTTAATGTT
3001 CGTTTTTCTT ATTTATATAT TTATACCAAT TGA
```

> PF11_0488 C-terminal-His

In yellow, 6xHis tag coding sequence.

```
   1 ATGAAATTAA ATTTGGATAA AAAAAGAGCA ATACTTGAAA AACGATTAGA TCATTTTAAT
  61 TTCCAAGAAA ACTCAGAATT CTCATTTTAT AATCCATTAA AAATAAATAT AAGAATGATG
 121 AACTTAATTG GAAGAGGAGG ATTTGCTGAA GTGTGGGAAG TTTTTGATTC TATCAATTTA
 181 GAAATGTATG CAGCCAAAAT TCATAAAATT GAACCAAGTA TGTCCAATGA AATAAAAAAT
 241 AAAATTATTC AAAGAGCAGA AAATGAAATA AATATACATA TACATTGTCA TAGACATATA
 301 TTTATTGTTA AATTAGAATT CTTTTTTGTA TTTGGTTCAG CAACAAATTT ATTAGTTGGA
 361 ATGGAATTAT GTGATATTGA TCTAGATAAA TATATTAAAT ATCATGGGCC AATTAATGAA
 421 CTTTTAGCTT TATGTTGGAT TAAACAAATA TTATTAGGCT TATTATATAT GAAAAATTTA
 481 CCAACTGGAA AAGTACACCA TTGTGATTTA AAACCTGCCA ACTTATTAAT CAAGGATGGA
 541 ATTATAAAAA TATCCGACTT TGGACTAGCC AAACTAATTT TACCAGATAC ACATCAATAT
 601 TACAATGGAG GTGGTACATT GTATTATCAA CCACCAGAAT GTTTAAAAAA TAAAAAAAAC
 661 CTTCTTATCA CAGATAAAAT TGATATCTGG TCATTGGGAT GCATTCTTTA TGAAATGCTC
```

```
 721 TTTTGTGAAA GACCTTTCCA ATTTAATTAC CTTGAAAAAT GTTCAAAAGA ATTATTAGTT
 781 AACAAAATGA AAAATGGATT AACCTATCCA AAAATTAATC AAAAAATTTC TAATGCTACT
 841 TTAAGTTACA TACAATATTT ACTAAATTTT GACTATGAAT TACGACCATC TATAGAAGAA
 901 GCCTTAAGCT ATCCAATTTT TAACTACTTT AATATACCAC TCGAGCACCA CCACCACCAC
 961 CACTGA
```

> *PF11_0488 C-terminal-HA-glmS-PF11_0488 3'UTR*

In blue, 3xHA tag coding sequence; in grey, glmS sequence; in yellow, shield mutation guide 2; in red, shield mutation guide 1; in lower case, non coding sequences.

```
   1 GAAATGTATG CAGCCAAAAT TCATAAAATT GAACCAAGTA TGTCCAATGA AATAAAAAAT
  61 AAAATTATTC AAAGAGCAGA AAATGAAATA AATATACATA TACATTGTCA TAGACATATA
 121 TTTATTGTTA AATTAGAATT CTTTTTTGTA TTTGGTTCAG CAACAAATTT ATTAGTTGGA
 181 ATGGAATTAT GTGATATTGA TCTAGATAAA TATATTAAAT ATCATGGGCC AATTAATGAA
 241 CTTTTAGCTT TATGTTGGAT TAAACAAATA TTATTAGGCT TATTATATAT GAAAAATTTA
 301 CCAACTGGAA AAGTACACCA TTGTGATTTA AAACCTGCCA ACTTATTAAT CAAGGATGGA
 361 ATTATAAAAA TATCCGACTT TGGACTAGCC AAACTAATTT TACCAGATAC ACATCAATAT
 421 TACAATGGAG GTGGTACATT GTATTATCAA CCACCAGAAT GTTTAAAAAA TAAAAAAAAC
 481 CTTCTTATCA CAGATAAAAT TGATATCTGG TCATTGGGAT GCATTCTTTA TGAAATGCTC
 541 TTTTGTGAAA GACCTTTCCA ATTTAATTAT TTAGAAAAAT GTTCAAAAGA ATTATTAGTT
 601 AACAAAATGA AAAATGGATT AACCTATCCA AAAATTAATC AAAAAATTTC TAATGCTACT
 661 TTAAGTTACA TACAATATTT ACTAAATTTT GACTATGAAT TACGACCATC TATAGAAGAA
 721 GCATTGAGCT ATCCAATTTT TAACTACTTT AATATACCAG GCGCGCCAGG CGGTGGATAC
 781 CCTTACGATG TGCCTGATTA CGCGTATCCC TATGACGTAC CAGACTATGC ATACCCTTAT
 841 GACGTTCCGG ATTATGCTCA CGGGGTGTAA GCGGCCGCGG TCTTGTTCTT ATTTTCTCAA
 901 TAGGAAAAGA AGACGGGATT ATTGCTTTAC CTATAATTAT AGCGCCCGAA CTAAGCGCCC
 961 GGAAAAAGGC TTAGTTGACG AGGATGGAGG TTATCGAATT TTCGGCGGAT GCCTCCCGGC
1021 TGAGTGTGCA GATCACAGCC GTAAGGATTT CTTCAAACCA AGGGGGTGAC TCCTTGAACA
1081 AAGAGAAATC ACATGATCTT CCAAAAAACA TGTAGGAGGG GACggcgcca gaaattatat
1141 atatatatca ttaaatattt tggggcacct atttttgta ttatataaat tggattattt
1201 cttataactc attgtaatac taatacatac ataaatatat atatatatat atatatacaa
1261 cattttgatt gttcttacat ttaaaaaagt aatatcattt tttattatat tcattaaaaa
1321 attttattca atattttctt atagatataa tatagattta tatatatata tatatatatt
1381 tatatattat aatattaata atattgaaat cattttata tattcatata catattatgt
1441 taactaatct cttccaaaag aaaatcaaaa aaaaaaaaaa aaaaatttaa cataaaaatt
1501 tatataatat aaacatacat tgttctttct ttatttttt tttttctcaa atgtatgaat
1561 aaaatatcat tttgataaat ttgaaggttt attatcctac tttttattgg gaaataaaaa
1621 tatcaaatgc gaacaatgaa ttctttaaaa ttc
```

> *PF11_0488 guide 1*

```
   1 GTTAAAAATT GGATAGCTTA
```

> *PF11_0488 guide 2*

```
   1 ATTCTTTTGA ACATTTTTCA
```

11. PF11_0488 aligment between Plasmodium species

In green, start codon of the C-terminal fragment.

P. falciparum	PF3D7_1148000
P. vivax	PVX_092985
P. chabaudi	PCHAS_0702300
P. berguei	PBANKA_0901100
P. yoelii	PY17X_0902500

```
P.falciparum      MYDYTEDNSLNPYLRQRIYHIRETLRNENELPLIDQIFKYELKKNFEDINDLLHYVNGII
P.vivax           MKDDNEDIISRNFLKQRIKNVKSILVKENEMPKIDQIFKYELKSNFTEINDLLYYVNGII
P.chabaudi        ----MTENDPSNFLKQRISNLKNILAKENEMPKIDQIFKYELKTNFTEINDLLHYMNGII
P.berghei         ----MTENDPSNFLKQRISNLKNILTKENEMPKIDQIFKYELKTNFTETNDLLHYMNGII
P.yoelii          ----MTENDPSNFLKQRISNLKNILTKENEMPKIDQIFKYELKTNFTETNDLLHYMNGII
                        :     :*:*** .::. * :***:* **********.** : ****:*:****

P.falciparum      YKGIDSFEKLTLLFTYDDKNDYDTTNLKQNDFIYLLRQKTKFRVKYKDENYIEYLRTNPL
```

```
P.vivax          YKNADTFQKLILLYSYDDNNDYNTSNVKQNTFVYLLKQKIKFREKYKNENYVDYLKTNPL
P.chabaudi       YKNLDTFQKLKLLYSYDDNNCYNNTGINQNTFLFLLKQKSKFREKYINENYMDYLRTNPL
P.berghei        YKNLDTFQKLKLLYSYDDKNCYNNSGINQNTFLFLLKQKSKFREKYINENYMEYLRTNPL
P.yoelii         YKNLDTFQKLKLLYSYDDNNCYNNSGINQNTFLFLLKQKSKFREKYVNENYIDYLRTNPL
                 **  *:*:** **::***:* *:.: ::** *::**:** *** ** :***::**:****

P.falciparum     IFIDTLNNLLIIPGINFEYRLHNFDTKNSKYFLKKSDTKVNSFYNPFFIRVKNTNLKKTH
P.vivax          IFIDTLNKLLIVPGISFEFKVYNFDEKVNKYFIRKSDIKSNTLYNPCFIKSRNGHFKNII
P.chabaudi       IFVDTLNQLLIIPGINFEFKVYNFDEKVNKFFVKKPDIKINSLYNPCFIKIKESNFKNIV
P.berghei        IFVDTLNQLLIIPGINFEFKVYNFDEKVNKFFVKKSDIKINSLYNPCFIKIKESNFKNIV
P.yoelii         IFVDTLNQLLIIPGINFEFKVYNFDEKVNKFFVKKPDIKINSLYNPCFIKIKESNFKNIV
                 **:****:***:***.**::::*** * .*:*::* * * *::*** **: :: .:*:

P.falciparum     KIYRRTAKNS-------------------------------------SSNKEHHIEHY
P.vivax          KVSRKADKSSRKSDKKSDQKGEKQNDKQSGKQSGQPNGKQSGQPNGKQSGQPNEQHIESY
P.chabaudi       KINRKVEKNPKKNEHQ---------------------------------------IEHY
P.berghei        KINRKVEKNYKKNEHQ---------------------------------------IEHY
P.yoelii         KINRKVEKNYKKNEHQ---------------------------------------IEHY
                 *: *:. *.                                               ** *

P.falciparum     FVKQNSIPLTKKLKTDDSTN-----------ADSNNTTIPINTICEENSSKEK-----TS
P.vivax          FEKKNTLQNSKM--------LNTNLVSKEDVNSKNEQHIDHDNPCSEEAKCELEITHIDD
P.chabaudi       FAKRNSVPSITQISKIECDSKEDNLISKDDLT------IATNSSCKND------DADLSS
P.berghei        FAKRNSVPSITQKSKTECDSKEDNVISKDDLT------IAINSSCKNY------DAELSS
P.yoelii         FAKRNSVPSITQKSKTECDSKEDNLISKDDLT------IPINSSCKNY------DPDLSS
                 * *:*::   .                          *  :. *.:           .

P.falciparum     NTQYNYTNHICEKPT---------------------NQNISIHSQTKYEPKKERRKRRS
P.vivax          DTDKKQNGEISRKMELTGDGNCK--------NDHNSAAPAAASDDEMKEGDKRERKKRKS
P.chabaudi       NNETKIYNEACDKGELDDSLNSIKQDSNSTTKKHSNESVNTIDDKEIKYDCRKEKKPRRS
P.berghei        NNETKIYNGICDKGELNDNLNTIKHDINNITKKHSNESINTIDNKGIKYECRKEKRARRS
P.yoelii         NNETKIYNEVCDKGELNGNLNTIKHDINNTTKKHSNESINTIDDKEIKYECRKEKRPRRS
                 :.: :     . *                                ..  *   ::*:: *:*

P.falciparum     LNTSTISDANNTDLGKNSKR--NISRCLKRKASQSNSTKQSKCEEDAQQEDEETTIEEEN
P.vivax          VNTTCILERNSSAQYNKKYKTNNTSNGVN------KESSETVCSNGLKGKAEDG------
P.chabaudi       FSISTNAESN------IK---------TN------TKKTKGNYNNSVKRKYDKV------
P.berghei        FSISTNPESN------KK---------TS------TKKNKGSYNSSVKRKYDKV------
P.yoelii         FSISTNAESN------NK---------TS------TKKTKGNCNSSVKRKYDKV------
                 .. :   : *       .          .      ....:   ..  : : :.

P.falciparum     KKMKEVEKEQMM-DKEKEIEKEKIKEESNLNEKNDQYNEDYGDNIDQEDYSSLNNYSSST
P.vivax          EGVEDVEVTEVTAEDEGEGEEEQED----------EEEEK----EREEEY---EYESSRN
P.chabaudi       SGCKKL----------KKGDEEEID----------ECEE--------EDD---EEGNSLN
P.berghei        SSCKKL----------KKGEEEEID----------EFEE--------EGD---EEDNSLN
P.yoelii         SSCKKL----------KKGEEEEID----------EFEE--------EGD---EEDNSLN
                 .  :.:           : ::*: .          : :*        *     :  .* .

P.falciparum     NSPKNTKSTYFFYDLINEYEIYINHAKCYIIFDIKSYYKNLDIMKKLKKNLEELKKPNNL
P.vivax          DDSGSGKSTHFFYNLVNEYEVYINNVKCYLVFDLMSCYKNLDIMKKLKKNLEELKKPNNV
P.chabaudi       NDGSTKQSIHTFYDLINEYEIYINNAKCYIIFDLKGYYKNLDIMKKLKKNLEDLKRPTND
P.berghei        NDGVPQKSIHTFYDLINEYEIYINNAKCYIIFDLKGYYKNLDIMKKLKKNLEDLKRPTND
P.yoelii         NDGVPPKSIHTFYDLINEYEIYINNAKCYIIFDLKGYYKNLDIMKKLKKNLEDLKRPTND
                 :.    :* : **:*:****:***..***::**: . ***************:**:*.*

P.falciparum     FKEITQKKTFKSSRDKMEFIKRFKKMIIPNFRLEKIRKQRNHLVIIELMSKIQNSLIIKR
P.vivax          FKEIINKKTFKSSRDKIEFIKRFKKMIIPNFRIEKIRKQRNHLIIIELMSKIQNSLIIKR
P.chabaudi       FKKIIDKKTFKSYRDKIEFVKRFKKFIIPNFRLEKIRKQRNHLIIVELMSKIQNRLIIKR
P.berghei        FKKIIDKKTFKSYRDKIEFVKRFKKFIIPNFRLEKIRKQRNHLIIVELMSKIQNRLIIKR
P.yoelii         FKKIIDKKTFKSYRDKIEFVKRFKKFIIPNFRLEKIRKQRNHLIIVELMSKIQNRLIIKR
                 **:* :****** ***:**:*****:******:**********:*:******** *****

P.falciparum     LYKELLEKVNIEELIKNMVVLFEKCVYNMKEEEIKNFYLRMIHTYFRSKDVKEIDFRKLI
P.vivax          LYQELTNKVNLHDLIKNAVQLFTKSVENMKNESVKKFYLSMINTYFQNNNLSAVDFKNLV
P.chabaudi       LNQELTNKVNLENLINDVLKMFSVFVENMKDENVKKFYTNMINTYFRNKNISSVDFKNLI
P.berghei        LNQELTNKVNLENLINDVLKMFSNFVENMKDENVKKFYMNMINIYFRNKNLSSIDFKNLI
P.yoelii         LNQELTNKVNLENLINDVLNMFSEFVENMKDENVKKFYTNMINTYFRNKNLSSVDFKNLI
                 * :** :***:.:**:: : :*   * ***:*.:*:**  **. **:.:::. :**::*:

P.falciparum     QQFKKIEEYRKNQEFYNLFQNDLNYLEKNKRECQEIDEKIHSLKYLILESILKEKQLERS
P.vivax          QLFKKKEEHLKNEEFYNLFQNDLNYLEKNKKQCDEIEAKINSLKYLILESILKEKQLERS
```

```
P.chabaudi        QLFKKKEEHEKSQEFYDLFQNDLNYLDKNKTQCDEIEEKISSLKYLILESILKEKQLERS
P.berghei         QLFKKKEEHEKNQEFYDLFQNDLNYLDKNKTQCDEIEEKISSLKYLILESILKEKQLERS
P.yoelii          QLFKKKEEHEKNQEFYDLFQNDLNYLDKNKTQRDEIEEKISSLKYLILESILKEKQLERS
                  * *** **: *.:***:*********:*** : :**: ** ******************

P.falciparum      VNRLLLNHEQSKYGYFYKIDQSDENNLDTTEYGKLLENFSKEPVNFYTILNKRNLDKHAY
P.vivax           VNRLLLNHEQSKYGYFYKVDQSDENTLDTTEYGKLLENFSKEPINFYTILNKRNLDKHAY
P.chabaudi        VNRLLLNHEQSKYGYFYKIDQSDENTLDTTEYGKLLENFSKEPINFYTILSKRNLDKHAY
P.berghei         VNRLLLNHEQSKYGYFYKIEQSDENTLDTTEYGKLLENFSKEPINFYTILSKRNLDKHAY
P.yoelii          VNRLLLNHEQSKYGYFYKIDQSDENTLDTTEYGKLLENFSKEPINFYTILSKRNLDKHAY
                  ******************::*****.*****************:******.*********

P.falciparum      HDIRNFNYKKKNNEEEQTKHVVNTALINNQANNNENKYAINKGEQNNIPLEQAKQVNNNN
P.vivax           HDIRNFNYKKKNNDEEALKHDATTSSPNQVNKS-IRTS-CENC-KLDAEGEEENQTEDKS
P.chabaudi        HDIRNFNYKKKDIDM--SKHDATNSSNNQGNYKKNKQF-LDNC-KFNMDCNQRKDENNNY
P.berghei         HDIRNFNYKKKNIDI--SKHDATNS----------------------------------
P.yoelii          HDIRNFNYKKKNIDI--SKHDATNSSINQGNYKRNKQF-LGNC-KFNMDCNQRKNENNNY
                  ***********: :    ** ...:

P.falciparum      YNNVKI-NGGDPHINNKNLNEEKHASQRIGKNEKEHIIKTNAQKEK--------------
P.vivax           KKNEHASSGAKDQKRDS---------NSSG----------NAKKSPNNQCANLKEKIKKG
P.chabaudi        NINGQITSSNNPENNDN---------NSSC-NIKDSVLITSDKKTNNSDCQII-------
P.berghei         --------SNNPENNDN---------NSSC-NIKDSVLITNDKKTNNNDCQII-------
P.yoelii          NINEQITSSNNPENNDN---------NSLC-NIKDSILVTSDKKTNNSDCQII-------
                         . . . .:.         :             . :*

P.falciparum      -EKEKENKISNIKEKQTPKDNNANKNDNINKEYYKCKIKQMNNEKKNDEEKEKNVVLKKK
P.vivax           INKNDNRNMPK-QEAQNGEKEPPD-NCNNQGDHYKGKIKKTNGDDKNQENN--------Q
P.chabaudi        -DNN---NMLN-CQKYIGQNKPQNENNYNTNEYYKCKIKKIQTDTSNEKNNEKNFVPKYD
P.berghei         -DN----NMFN-CPKKIAQNQPQNENNYNTDEYYKCKIKKIQTDTPNEKNNENNSVLEYD
P.yoelii          -DNNNNNNMFN-CQKNIGQNQPRNENNYNTNEYYKCKIKKIQTDTPNEKNNENNSVLEYD
                   ::    :: :       :.:  : *     ::** ***: : :  *::::        .

P.falciparum      K--KYNTFNLFPKNNKNDSNESYDKNYFRKEEKLLSTINLRKRLAEIDKNPYSK--SDDN
P.vivax           HNKKINCSQIV-----------------------ESKNTQNSSTTINNGKKNEACKEEN
P.chabaudi        FDKKQNCAQID-----------------------ENKSIDKSVTTNDSDPVTQHCSHQN
P.berghei         FDKKLNCLQID-----------------------ENKNIDKSVTTNDSDVVNQQCSRQN
P.yoelii          FDKKRNCPQID-----------------------ENKNIDKSVTTNDSDAVNQQCSHQN
                     * *  ::                         .. .  :  :  :.   .:  . :*

P.falciparum      NNNNDNDNNNNSNNNNNNDNDNNNNSNNNNNDNNDNNELSGEGRLSSTGMYKTEEYLNEI
P.vivax           GRNKKKDSAKNCTHKGKKEA-AFKKS----SSCENKKQANFKEKYKNHEEEMPSEYETEC
P.chabaudi        TSQIN---GCIDTNKNIKES-HFKKS----NSYEDKRQINPKTKHKFERDDPVIECGYEL
P.berghei         TRQIN---FCIDTNKNIKES-HLKKS----NSYEEKRQINPKAKHKFEREDPIIECGYEP
P.yoelii          TSQIN---CCIDTNKNIKES-HFKKS----NSYEDKRQINPKAKHKFERDDPVIECGYEP
                    : .       ..:  ::    ::*    .. :::.: . : : .        *   *

P.falciparum      KKDIVRCICEKKNYNFINEKQEKINNEIFYKVFEQYPYSFFSKSVKNYKIILNENEEESE
P.vivax           ERDIIRSTCEKKNFTFANDKQEKINNEIFYKVFEQYPYSFFSKSVKNYNLILNENKEESE
P.chabaudi        ETDIIRSICEKKKFTFANEKQEKINNEIFYKVFEQYPYSFFSKSVKNYNAILNENEEESE
P.berghei         ETDIIRSICEKKKFTFANEKQEKINNEIFYKVFEQYPYSFFSKSVKNYNAILNENEEESE
P.yoelii          ETDIIRSICEKKKFTFANEKQEKINNEIFYKVFEQYPYSFFSKSVKNYNAILNENEEESE
                  : **:*. ****::.* *:*****************************: *****:****

P.falciparum      LSWLTMLKKKSHNRSILPPSRDTFRDGTHFSNCRATEHTLKFFLSLLTLLRKGPIDLNLK
P.vivax           LSWLTMLKKKTHNKSILPPSRDTFRDGTHFSNCRATEHTLKFFLSLLSLLTKSDIDINLK
P.chabaudi        LSWLTMLKKKSHNRSILPPSRDTFRDGTHFSNCRATEHTLKFFLSLLSLLTKGDIDINLK
P.berghei         LSWLTMLKKKSHNRSILPPSRDTFRDGTHFSNCRATEHTLKFFLSLLSLLKKGDIDINLK
P.yoelii          LSWLTMLKKKSHNRSILPPSRDTFRDGTHFSNCRATEHTLKFFLSLLSLLTKGDIDINLK
                  **********:**:**********************************:** *. **:***

P.falciparum      KYLKKNIQFLNTELFSMKLNLDKKRAILEKRLDHFNFQENSEFSFYNPLKINIRMMNLIG
P.vivax           QYLKKNIQFLNTELFSMKLNLDKKRAILEKRLDHFNFQENSEFSFYNPLKMNIRMMNLIG
P.chabaudi        KYLKKNIQFLNTELFSMKLNLDKKRAILEKRLDHFNFQENSEFSFYNPLKMNIRMMNLIG
P.berghei         KYLKKNIQFLNTELFSMKLNLDKKRAILEKRLDHFNFQENSEFSFYNPLKMNIRMMNLIG
P.yoelii          KYLKKNIQFLNTELFSMKLNLDKKRAILEKRLDHFNFQENSEFSFYNPLKMNIRMMNLIG
                  :************************************************:*********

P.falciparum      RGGFAEVWEVFDSINLEMYAAKIHKIEPSMSNEIKNKIIQRAENEININIHIHCHRHIFIVK
P.vivax           RGGFAEVWEVFDSINLEMYAAKIHKIEPSMTNEIKNKIIQRAENEININIHIHCHRHIFIVK
P.chabaudi        RGGFAEVWEVFDSINLEMYAAKIHKIEPSMTNEIKNKIIQRAENEININIHIHCHRHIFIVK
```

```
P.berghei          RGGFAEVWEVFDSINLEMYAAKIHKIEPSMTNEIKNKIIQRAENEININIHIHCHRHIFIVK
P.yoelii           RGGFAEVWEVFDSINLEMYAAKIHKIEPSMTNEIKNKIIQRAENEININIHIHCHRHIFIVK
                   ****************************:****************************

P.falciparum       LEFFFVFGSATNLLVGMELCDIDLDKYIKYHGPINELLALCWIKQILLGLLYMKNLPTGK
P.vivax            LEFFFVFGSATNLLVGMELCDIDLDKYIKYHGPINELLALCWVKQILLGLLYMKTLPTGK
P.chabaudi         LEFFFVFGSATNLLVGMELCDVDLDKYIKYHGPINELLALSWIKQILLGLLYMKNLPTGK
P.berghei          LEFFFVFGSATNLLVGMELCDVDLDKYIKYHGPINELLALSWIKQILLGLLYMKNLPTGK
P.yoelii           LEFFFVFGSATNLLVGMELCDVDLDKYIKYHGPINELLALSWIKQILLGLLYMKNLPTGK
                   ********************:******************.*:***********.*****

P.falciparum       VHHCDLKPANLLIKDGIIKISDFGLAKLILPDTHQYYNGGGTLYYQPPECLKNKKNLLIT
P.vivax            VHHCDLKPANLLIKDGIIKISDFGLAKLILPDTYQYYNGGGTLYYQPPECLRNKKNLLIT
P.chabaudi         VHHCDLKPANLLIKDGIIKISDFGLAKLILPDTYQYYNGGGTLYYQPPECLKPKRNLLIT
P.berghei          VHHCDLKPANLLIKDGIIKISDFGLAKLILPDTYQYYNGGGTLYYQPPECLKPKRNLLIT
P.yoelii           VHHCDLKPANLLIKDGIIKISDFGLAKLILPDTYQYYNGGGTLYYQPPECLKPKRNLLIT
                   *********************************:*****************: *:*****

P.falciparum       DKIDIWSLGCILYEMLFCERPFQFNYLEKCSKELLVNKMKNGLTYPKINQKISNATLSYI
P.vivax            DKIDIWSLGCILYEMLFCERPFQFNYLEKCSKELLVNKMKRGLSYPKINQQISEVTLNYI
P.chabaudi         DKIDIWSLGCILYEMIFCERPFQFNYLEKCSKELLVNKMKRGLSYPKINQHISKITLNYI
P.berghei          DKIDIWSLGCILYEMIFCERPFQFNYLEKCSKELLVNKMKRGLSYPKINQHISKITLNYI
P.yoelii           DKIDIWSLGCILYEMIFCERPFQFNYLEKCSKELLVNKMKRGLSYPKINQHISKITLNYI
                   ***************:************************.**:******:**: **.**

P.falciparum       QYLLNFDYELRPSIEEALSYPIFNYFNIP
P.vivax            QYLLNFDYEFRPSIEEALAYPIFNFFRIP
P.chabaudi         EYLLNFDHESRPSIEEALSYPIFNYFNIP
P.berghei          EYLLNFDHECRPSIEEALSYPIFNYFNVP
P.yoelii           EYLLNFDHECRPSIEEALSYPIFNYFNIP
                   :******:* ********:*****:*.:*
```

Appendix III: PFE0825w uptake conditions

Substrate	PFE0825w variant	Concentration (µM)*	pH	Temperature (°C)
TEA	V1-his	20	7.4	19
			6	
			5	
			6	25
		20 + 200	6	25
		20 + 20	6	19
		20 + 60		
		20 + 140		
		20 + 200		
		20 + 300		
	V1	50+150	6	25
		50+150	7.4	25
		50+150	6	30
	V0-his	20	6	19
		20+480	7.4	
		20		
		50		
		200		
		250		
		500		
		50	7.4	25
		100		
		250		
		500		
	V0	50+150		
		50+150	6	25
		50+150	6	30
MPP	V1-his	100	7.4	25
	V1		6	30
	V0			

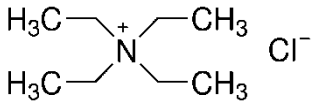
Choline	V1-his	10	7.4	19
		50		
		150		
		10	6	
		50		
		150		
		10	5	
		50		
		150		
		10	5	25
	V0-his	10	7.4	19
		50		
		150		
		10	6	
		50		
		150		
		10	5	
		50		
		150		
T3	V0	200	6	30
	V1			
CQ	V1-his	10	6	19

* All the concentrations for TEA correspond to [^{14}C]-TEA + TEA; the concentrations for Choline, MPP and CQ correspond to the concentration of unlabeled compound and the concentration for T3 correspond to the concentration of radiolabelled compound. The uptake buffer in the case of Choline, MPP and CQ only contains tracing amounts of radiolabelled compounds: CQ 40 nM; Choline 12 nM; MPP 25 nM.

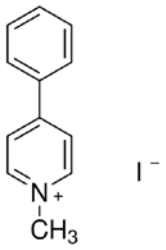
Appendix IV: Compounds structures

> Compounds used on uptake experiments

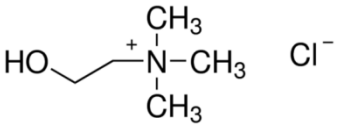
TEA



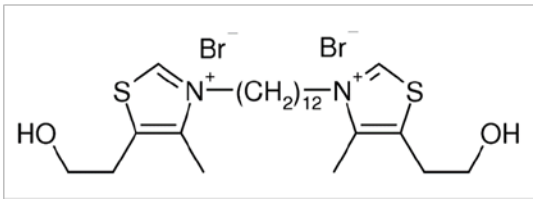
MPP



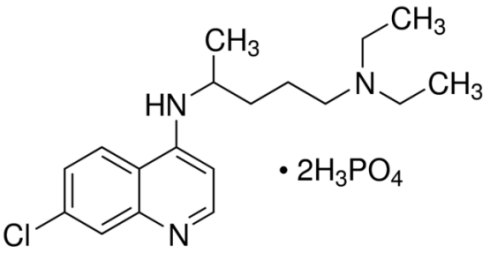
Choline



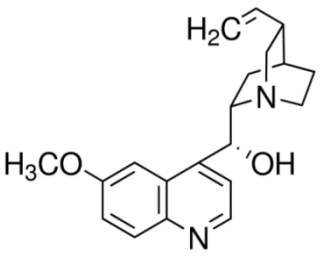
T3



CQ

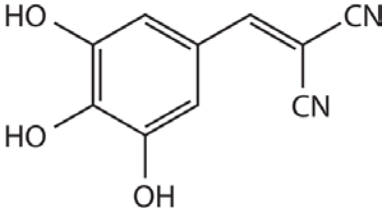


QN

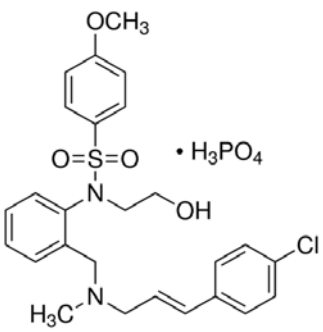


> Compounds used on the CQ accumulation screen

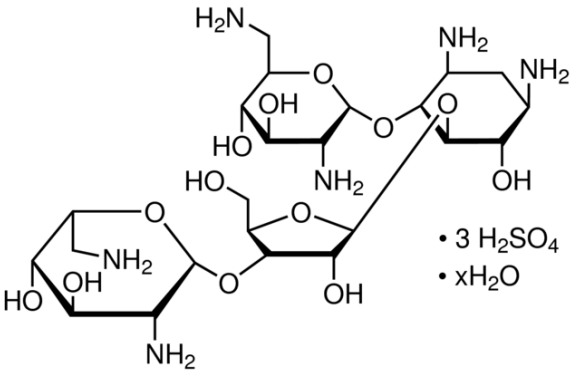
Tyrphostin A25



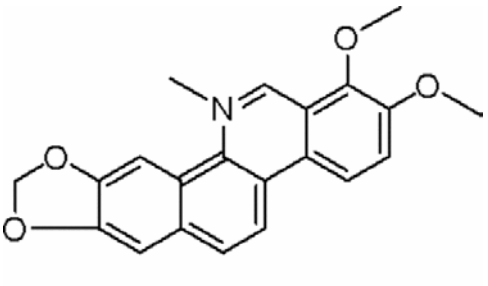
KN-93



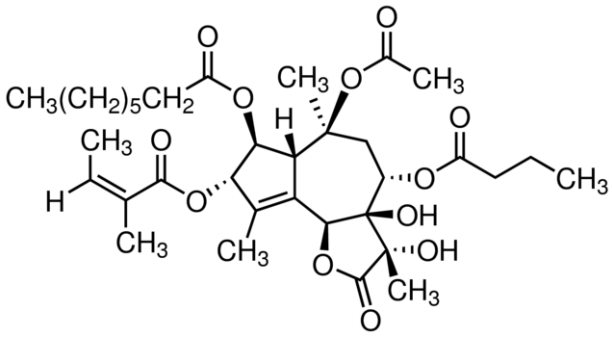
Neomycin



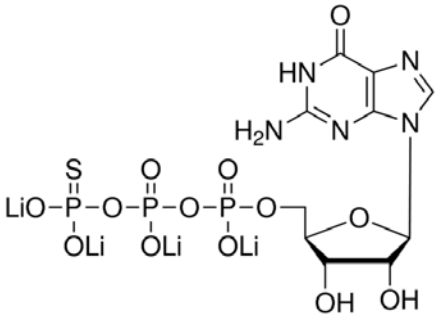
Chelerythrine



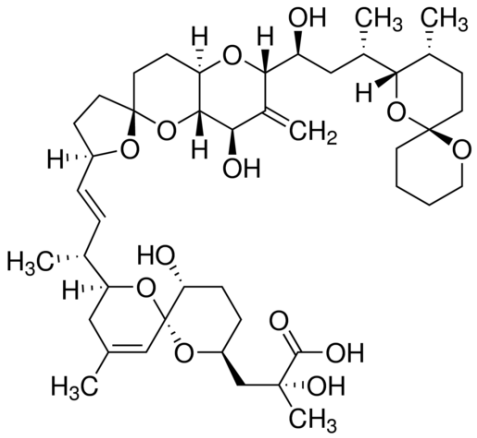
Thapsigargin



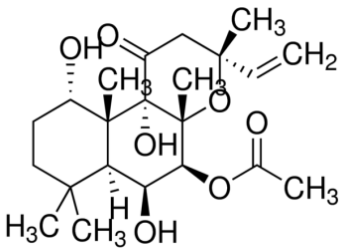
GTP-gamma-S



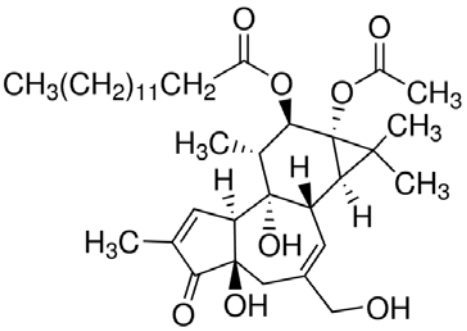
Okadaic acid



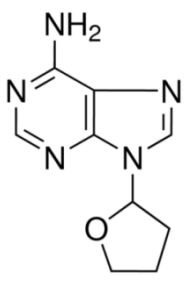
Forskolin



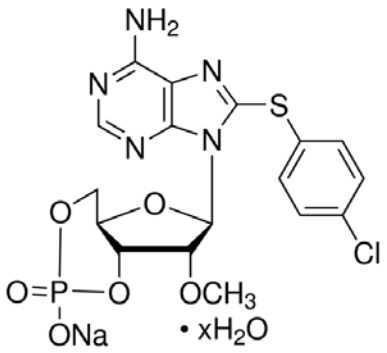
PMA



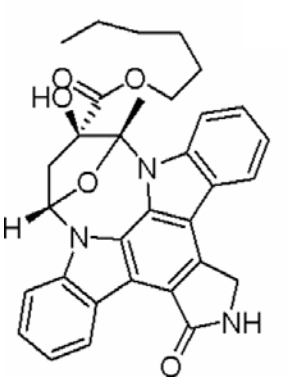
SQ22,536



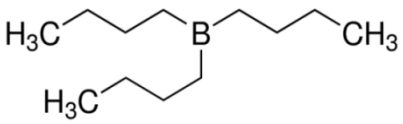
CTP-cAMP



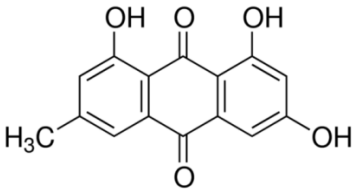
KT-5720TBB



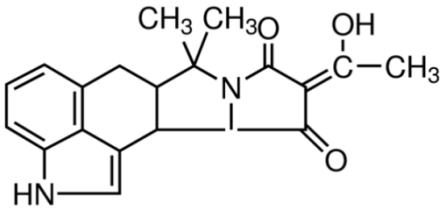
TBB



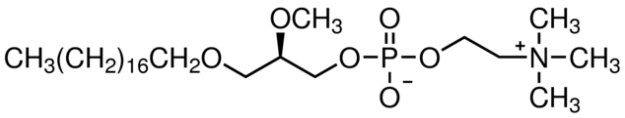
Emodin



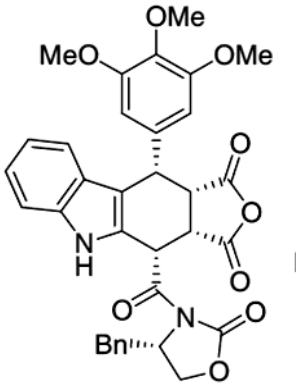
CPA



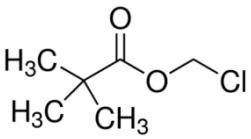
ET-18-OCH3



W16

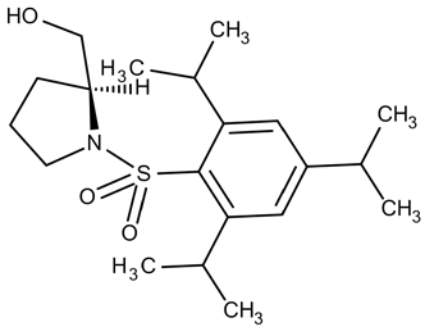


POM

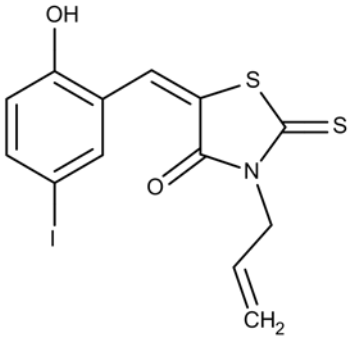


> ML-7 Analogs

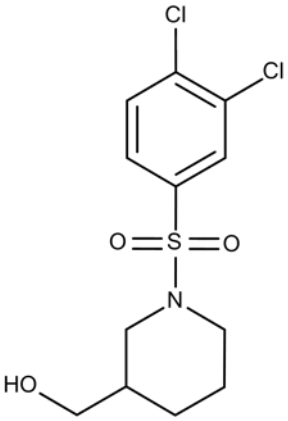
K100018565



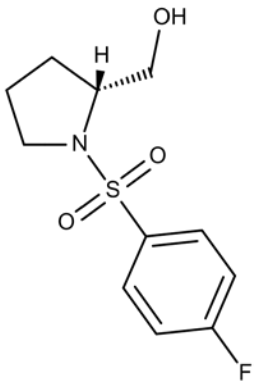
K100025569



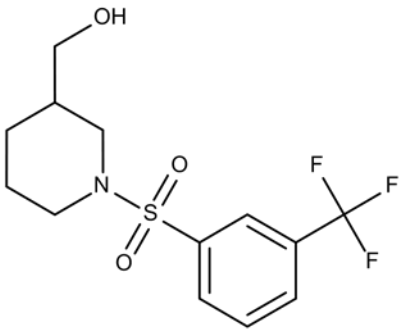
K100027117



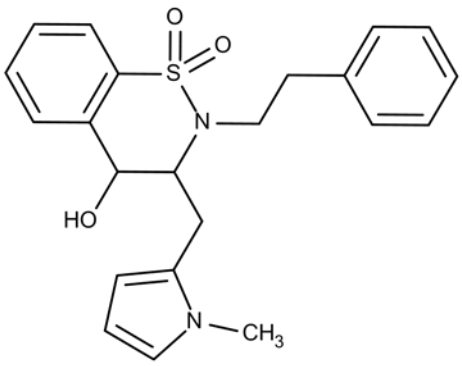
K100027120



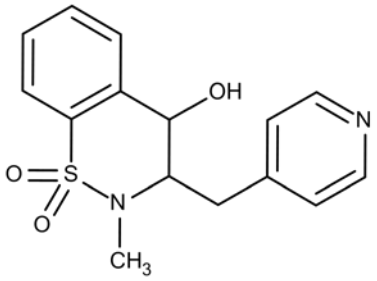
K100027139



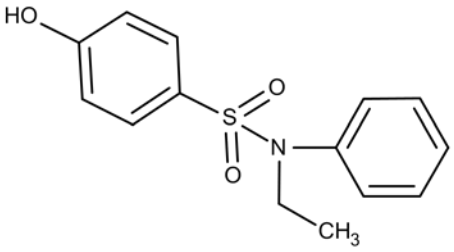
K20034774



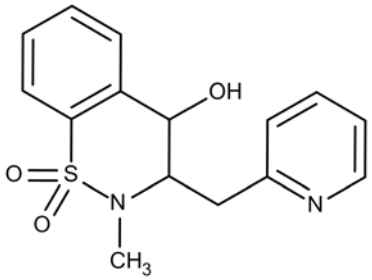
K20034808



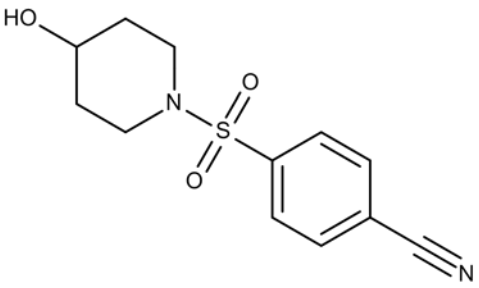
K20035017



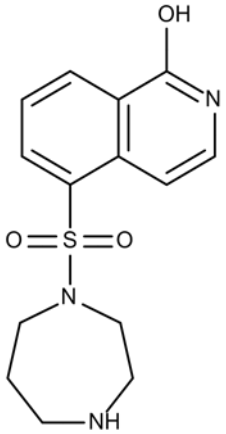
K20035244



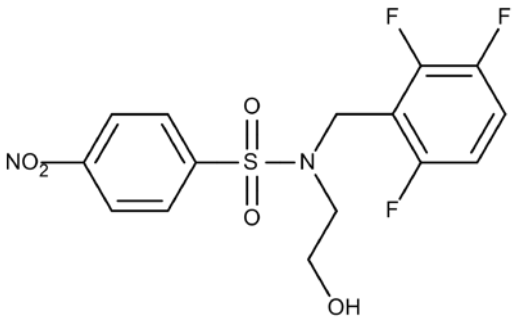
K20037423



K20037526



K20037657



Appendix V: Y2H prey sequences

1. PF3D7_1427900 - PF14_0257 + PF3D7_0707300 - MAL7P1.208 (2x)

Bait: PfCRT N-terminal

In frame? Two sequences on the same plasmid.

PF3D7_1427900 is in frame and PF3D7_0707300 is not.

Sequence:

ORF 1 (In frame)

EEEEYEEDNTLKNFYEADFKDEDDEDEEFVPNDNEDDDEDDEMKDEVMIMQIVLLRQTIMKITMIKMKKKKNIMI
KIMIMDITF

ORF 2

RRRRRI*RR*YFKKFL*S*L*R*R**R*RICTQ***R**RR*RDER*SDDYADSFIETDHYENNDDKNEEEEEYNDQD
NDYGYNFLETDEYDDSEEYDYDDKEYGESFLEKEEGEEMKDEEMKDEEMEDVEMKDEEMKDEEMKYDEMKNEE
MKYDEMKDEVM

Full plasmid sequenced. The underlined sequences are the ones found in the BLAST search.

BLAST ORF 1:

	Score	E
PF3D7_1427900 | organism = Plasmodium_falciparum_3D7 |	35.8	4e-04
PF3D7_1468100 | organism = Plasmodium_falciparum_3D7 |	23.5	8.7

>PF3D7_1427900

Score = 35.8 bits (81), Expect = 4e-04, Method: Compositional matrix adjust.Identities = 29/29 (100%), Positives = 29/29 (100%), Gaps = 0/29 (0%).

```
Query  9    DNTLKNFYEADFKDEDDEDEEFVPNDNED  37
            DNTLKNFYEADFKDEDDEDEEFVPNDNED
Sbjct  186  DNTLKNFYEADFKDEDDEDEEFVPNDNED  214
```

No predicted domains by Prosite.

Annotation: Conserved protein, unknown function.

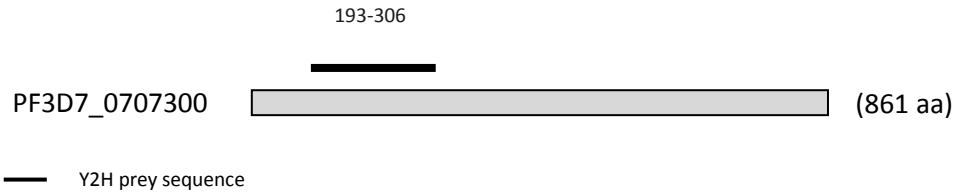
BLAST ORF 2:

	Score	E
PF3D7_0707300 | organism = Plasmodium_falciparum_3D7 |	113	2e-28
PF3D7_0406500 | organism = Plasmodium_falciparum_3D7 |	40.0	3e-04
PF3D7_0405200 | organism = Plasmodium_falciparum_3D7 |	32.7	0.055
PF3D7_0918700 | organism = Plasmodium_falciparum_3D7 |	32.7	0.074
PF3D7_1140700 | organism = Plasmodium_falciparum_3D7 |	32.7	0.078
PF3D7_1251600 | organism = Plasmodium_falciparum_3D7 |	31.2	0.22
PF3D7_1410300 | organism = Plasmodium_falciparum_3D7 |	31.2	0.24
PF3D7_1149000 | organism = Plasmodium_falciparum_3D7 |	30.0	0.46
PF3D7_1313500 | organism = Plasmodium_falciparum_3D7 |	30.0	0.53
PF3D7_0506500 | organism = Plasmodium_falciparum_3D7 |	29.3	0.97
PF3D7_1123100 | organism = Plasmodium_falciparum_3D7 |	27.3	4.1
PF3D7_1032700 | organism = Plasmodium_falciparum_3D7 |	26.9	4.2
PF3D7_1359700 | organism = Plasmodium_falciparum_3D7 |	26.9	5.6
PF3D7_1133700 | organism = Plasmodium_falciparum_3D7 |	26.2	8.8

>PF3D7_0707300

Score = 113 bits (283), Expect = 2e-28, Method: Compositional matrix adjust. Identities = 112/114 (98%), Positives = 113/114 (99%), Gaps = 0/114 (0%)

```
Query  49   DDYADSFIETDHYENNDDKNEEEEEYNDQDNDYGYNFLETDEYDDSEEYDYDDKEYGESF  108
            DDY DSFIETDHYENNDDKNEEEEEYNDQDNDYGYNFLETDEYDDSEEYDYDDKEYGESF
Sbjct  193  DDYTDSFIETDHYENNDDKNEEEEEYNDQDNDYGYNFLETDEYDDSEEYDYDDKEYGESF  252

Query  109  LEKEEGEEMKDEEMKDEEMEDVEMKDEEMKDEEMKYDEMKNEEMKYDEMKDEVM  162
            LEKEEGEEMKDEEMKDEEM+DVEMKDEEMKDEEMKYDEMKNEEMKYDEMKDEVM
Sbjct  253  LEKEEGEEMKDEEMKDEEMKDVEMKDEEMKDEEMKYDEMKNEEMKYDEMKDEVM  306
```



No predicted domains by Prosite.

Annotation: Rhoptry-associated membrane antigen (RAMA).

2. PF3D7_0609000 - PFF0445w -MAL6P1.93

Bait: Putative Calmodulin Binding Site

In Frame? Yes

Sequence:

TINNTNTGGNIFSSPLSNSLNQGITNANANTITNTNANNIFNISSNSALLNNSSNKLFGTTTNTASSSNLLGNNNISSGMFSPLSNNINNKPNLFSGANQNNLFSNTNMSSSPSLSLNNTTNTIGGNINSSGQNFIQNQNNILTNQTLSNSIYNNNSNLNSNNLLLPGQQQNNTSPFLTNMGTNASSPTSSIFNQSKDLISSNNLNIGTSTTNIFGTTSSNNMNNMNSMNSMNSMNNMNSMNSMNSMNSMNNMNSMNSLFLGLQQQTQSTTTTT

The full insert was not sequenced. The underlined sequence is the ones found in the BLAST search.

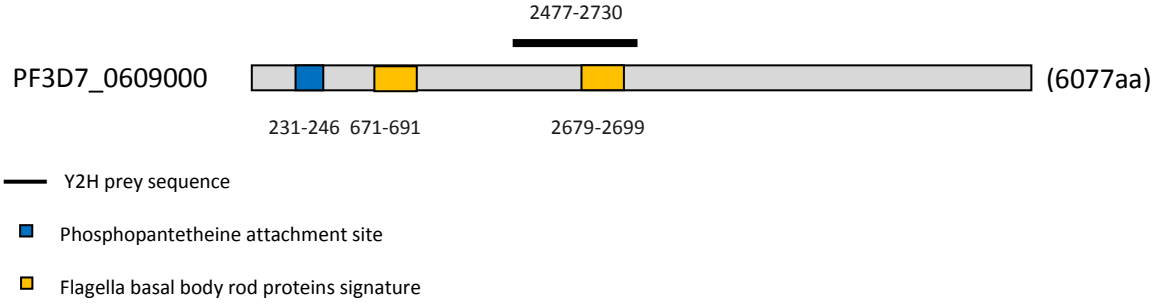
BLAST:

	Score	E
PF3D7_0609000 | organism = Plasmodium_falciparum_3D7 |	310	9e-95
PF3D7_1228800 | organism = Plasmodium_falciparum_3D7 |	32.3	0.14
PF3D7_0629400 | organism = Plasmodium_falciparum_3D7 |	28.1	2.7

>PF3D7_0609000

Score = 310 bits (793), Expect = 9e-95, Method: Compositional matrix adjust. Identities = 263/302 (87%), Positives = 265/302 (88%), Gaps = 22/302 (7%)

```
Query  8     GGNIFSSPLSNSLNQGITNANANTITNTNANNI--FNISSNSALLNNSSNKLFGTTTNTA  65
             GGNIFSSPLSNSLNQGITNANAN  T TN N    FNISSNSALLNNSSNKLFGTTTNTA
Sbjct  2477  GGNIFSSPLSNSLNQGITNANANANTITNTNANNIFNISSNSALLNNSSNKLFGTTTNTA  2536

Query  66    SSSNLLGNNNISSGMFSPLSNNINNKPNLFSGANQNNLFSNTNMSSSPSLSLNNTTNTIG  125
             SSSNLLGNNNISSGMFSPLSNNINNKPNLFSGANQNNLFSNTNMSSSPSLSLNNTTNTIG
Sbjct  2537  SSSNLLGNNNISSGMFSPLSNNINNKPNLFSGANQNNLFSNTNMSSSPSLSLNNTTNTIG  2596

Query  126   GNINSSGQNFIQNQNNILTNQTLSNSIYNNNSNLNSNNLLLPGQQQNNTSPFLTNMGTNA  185
             GNINSSGQNFIQNQNNILTNQTLSNSIYNNNSNLNSNNLLLPGQQQNNTSPFLTNMGTNA
Sbjct  2597  GNINSSGQNFIQNQNNILTNQTLSNSIYNNNSNLNSNNLLLPGQQQNNTSPFLTNMGTNA  2656

Query  186   SSPTSSIFNQSKDLISSNNLNIGTSTTNIFGTTSSNNMNNMNSMNSMNSMNNMNSMNSMN  245
             SSPTSSIFNQSKDLISSNNLNIGTSTTNIFGTTSS               NNMN+MNSMN
Sbjct  2657  SSPTSSIFNQSKDLISSNNLNIGTSTTNIFGTTSS---------------NNMNNMNSMN  2701

Query  246   SMNSMNNMNSMNSLFLGLQQQTQSTTTTT  274
             SMNSMNNMNSMNSLFLGLQQQTQSTTTTT
Sbjct  2702  SMNSMNNMNSMNSLFLGLQQQTQSTTTTT  2730
```



Annotation: Conserved Plasmodium protein, unknown function.

3. PF3D7_0106900 - PFA0340w

Bait: OUT loop

In Frame? No

Sequence:

GKKRKRKK*TLFFFFPCI*DLFKYT*YFFMYLMMIFSFYHIC*INMLIFIYLFFFFL*EWHKYMHTQKKKKKNNNLERMHFVHTFIRCVLLIYFIKWNGYNFHMLKRQFFKNGKNIIERSIRKCKKNNFSKSYHSIVYIKNGVTQYMCKNKKRGRGEQKKNIIINNKYLFLLNNFIDKNKDKTYLSTSLERKYLKNQKDDAHKIWKNRIKNYKSINIYMSKIEEKSTKEIENKDDILNKDNINNKHIYDNNKENDIFYKYNTK

Full insert sequenced. The underlined sequence is the one found in the BLAST search.

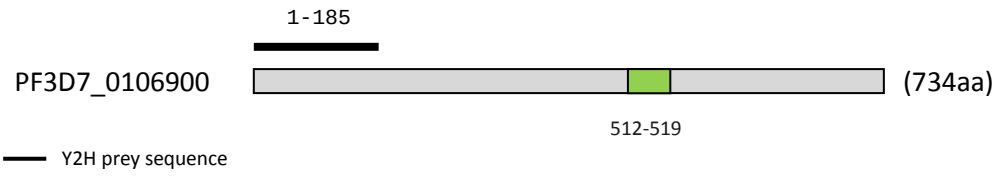
BLAST:

	Score	E
PF3D7_0106900 | organism = Plasmodium_falciparum_3D7 |	360	1e-119
PF3D7_1325500 | organism = Plasmodium_falciparum_3D7 |	35.4	0.010

>PF3D7_0106900

Score = 360 bits (925), Expect = 1e-119, Method: Compositional matrix adjust. Identities = 184/185 (99%), Positives = 184/185 (99%), Gaps = 0/185 (0%)

```
Query  79   MHFVHTFIRCVLLIYFIKWNGYNFHMLKRQFFKNGKNIIERSIRKCKKNNFSKSYHSIVY  138
            MHFVHTFIRCVLLIYFIKWNGYNFHMLKRQFFKNGKNIIERSIRKCKKNNFSKSYHSIVY
Sbjct  1    MHFVHTFIRCVLLIYFIKWNGYNFHMLKRQFFKNGKNIIERSIRKCKKNNFSKSYHSIVY  60

Query  139  IKNGVTQYMCKNKKRGRGEQKKNIIINNKYLFLLNNFIDKNKDKTYLSTSLERKYLKNQK  198
            IKNGVTQYMCKNKKRGRGEQKKNIIINNKYLFLLNNFIDKNKDKTYLSTSLERKYLKNQK
Sbjct  61   IKNGVTQYMCKNKKRGRGEQKKNIIINNKYLFLLNNFIDKNKDKTYLSTSLERKYLKNQK  120

Query  199  DDAHKIWKNRIKNYKSINIYMSKIEEKSTKEIENKDDILNKDNINNKHIYDNNKENDIFY  258
            DDAHKIWKNRIKNYKSINIYMSKIEEKSTKEIENKDDILNKDNINNKHIYDNNKENDIF
Sbjct  121  DDAHKIWKNRIKNYKSINIYMSKIEEKSTKEIENKDDILNKDNINNKHIYDNNKENDIFN  180

Query  259  KYNTK  263
            KYNTK
Sbjct  181  KYNTK  185
```



Annotation: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, putative (IspD).

4. PF3D7_0907000 - PFI0336w

Bait: Putative Calmodulin Binding Site

In Frame? No

Sequence:

CSYIFLVFSFVLSV*K*KKMSNDQDLKSSFLQDLKEYSTNDDKKFPEVLKNYITQNIEDQNEAERFLKEFNDSYLKEMN
LDELELLCSMILKKKNLSAN*GGTNKTIKIIINKLYKLN*NKINTYIYHIYHTIHFPYIFFFLFFFFIFFFFFIFYFFSYFLFFHIL
FFFIFYF*IFIVR*MLNICFIYS*DRESLSCFILFDI*FMTFS*YS*IS*F*FFKI*NI

Full insert was sequenced. The underlined sequences are the ones found in the BLAST search.

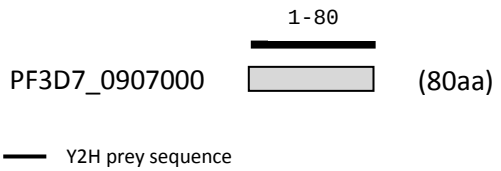
BLAST:

	Score	E
PF3D7_0907000 | organism = Plasmodium_falciparum_3D7 |	156	7e-51
PF3D7_1034300 | organism = Plasmodium_falciparum_3D7 |	25.4	1.9
PF3D7_1136100 | organism = Plasmodium_falciparum_3D7 |	25.4	1.9
PF3D7_1220300 | organism = Plasmodium_falciparum_3D7 |	24.6	3.1
PF3D7_0405700 | organism = Plasmodium_falciparum_3D7 |	23.9	5.4
PF3D7_1422700 | organism = Plasmodium_falciparum_3D7 |	23.9	6.5
PF3D7_1021900 | organism = Plasmodium_falciparum_3D7 |	23.5	9.6
PF3D7_1122900 | organism = Plasmodium_falciparum_3D7 |	23.5	7.7

>PF3D7_0907000

Score = 156 bits (394), Expect = 7e-51, Method: Compositional matrix adjust. Identities = 80/80 (100%), Positives = 80/80 (100%), Gaps = 0/80 (0%)

```
Query  3   MSNDQDLKSSFLQDLKEYSTNDDKKFPEVLKNYITQNIEDQNEAERFLKEFNDSYLKEMN  62
           MSNDQDLKSSFLQDLKEYSTNDDKKFPEVLKNYITQNIEDQNEAERFLKEFNDSYLKEMN
Sbjct  1   MSNDQDLKSSFLQDLKEYSTNDDKKFPEVLKNYITQNIEDQNEAERFLKEFNDSYLKEMN  60

Query  63  LDELELLCSMILKKKNLSAN  82
           LDELELLCSMILKKKNLSAN
Sbjct  61  LDELELLCSMILKKKNLSAN  80
```



No predicted domains by Prosite.

Annotation: conserved Plasmodium protein, unknown function

5. PF3D7_0919800 - PFI09700c

Bait: Putative Calmodulin Binding Site

In Frame? No – Inverted sequence

Sequence:

IVIIKTFHEEKNKRSISYYKYIRKSLSYVLIRSATSFNYTPFKENKIDKVQNWKI*YPVFHSRGGKNI*FEGYTK*CK*CE*
CE*CKYCE*YK*CKRNK**FV*K*Y**V*RDNM*TSKK**CQ*YCLAE**T*T***IYFDMSTSYNNNHNNNIVDHL
HDRDLKIFQKINEINYSTVIPSYPDIT*KNSLNLVNSQSSRKDGLSSDCDKKNNNQFKKKNEHGRGNEKEK

Poor sequence quality. The underlined sequence is the one found in the BLAST search.

BLAST:

	Score	E
PF3D7_0919800 | organism = Plasmodium_falciparum_3D7 |	121	4e-31

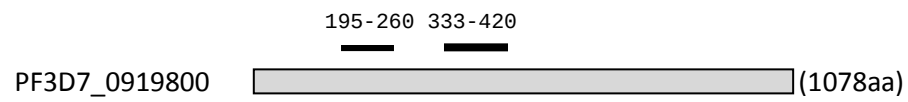
>PF3D7_0919800

Score = 121 bits (303), Expect = 4e-31, Method: Compositional matrix adjust. Identities = 79/88 (90%), Positives = 82/88 (93%), Gaps = 0/88 (0%)

```
Query  139  IYFDMSTSYNNNHNNNIVDHLHDRDLKIFQKINEINYSTVIPSYPDIT*KNSLNLVNSQS  198
            + FDMSTSYNNNHNNNIVDHLHD DLKI+QKINEINYSTVIPSYPDIT KNSLNL NSQS
Sbjct  333  LSFDMSTSYNNNHNNNIVDHLHDSDLKIYQKINEINYSTVIPSYPDITYKNSLNLANSQS  392

Query  199  SRKDGLSSDCDKKNNNQFKKKNEHGRGN  226
            S KD LSSDCDKKNNNQ+KKKNEHGRGN
Sbjct  393  SLKDDLSSDCDKKNNNQYKKKNEHGRGN  420
```

Score = 47.0 bits (110), Expect = 2e-06, Method: Compositional matrix adjust. Identities = 34/67 (51%), Positives = 40/67 (60%), Gaps = 4/67 (6%)

```
Query  9    EEKNKRSISYYKYIRKSLSYV--LIRSATSFNYTPFKENKIDKVQNWK-I*YPVFHSRGG  65
            +E  ++SISYYKYI KSLSYV  L +   + N T FKENK DKVQN + I   V    GG
Sbjct  195  DENKQKSISYYKYISKSLSYVFNLAQPLVTTN-TTFKENKNDKVQNLEDIISDVSFKGGG  253

Query  66   KNI*FEG  72
                F G
Sbjct  254  STYNFSG  260
```



No predicted domains by Prosite.

Annotation: TLD domain containing protein

6. PF3D7_1033200 - PF10_0323

Bait: Putative Calmodulin Binding Site

In Frame? No

Sequence:

STFFFFFFFFFFSFLFLLFFFFFLFF*F*F*F*FFFKMKVGKIFFLLNILVVCHFIISCLCRNGQTTRGNLLALKAIEQDLQQKKNRKRNLILYSLGSAALIAALVVTGIGLNMYMKKKNVDSEVQEIIDEKDEKVKEKPAEKKKTTVKIVSKRVPVKSKSSNGKSKARTVNSEVSPKLDDEKKEDLLKFNDNDLLLAAESLKELNPKYDENTQGNDSFKNINEPRKLASFSLYDALADASEQNKNKDAESSTGQIPTPTESSHGISDGKKDTSTNDMDPLNPYGSSKRNSSENKPTSESKGTTPESNFDSKTPEIKEINEPIIVPSYYPTTGPNPNTHGPP

The full insert was not sequenced. The underlined sequence is the one found in the BLAST search.

BLAST:

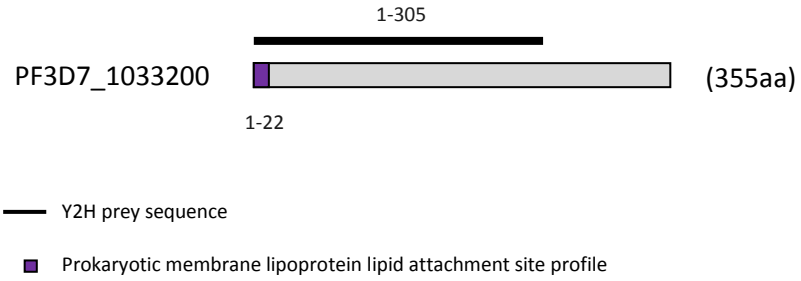
	Score	E
PF3D7_1033200 | organism = Plasmodium_falciparum_3D7|	592	0.0
PF3D7_1302200 | organism = Plasmodium_falciparum_3D7|	36.2	0.007
PF3D7_1401400 | organism = Plasmodium_falciparum_3D7|	35.0	0.005
PF3D7_1001500 | organism = Plasmodium_falciparum_3D7|	32.3	0.046
PF3D7_1102800 | organism = Plasmodium_falciparum_3D7|	32.3	0.030
PF3D7_0512500 | organism = Plasmodium_falciparum_3D7|	29.6	1.2
PF3D7_1016900 | organism = Plasmodium_falciparum_3D7|	26.2	5.4

>PF3D7_1033200

Score = 592 bits (1527), Expect = 0.0, Method: Compositional matrix adjust. Identities = 304/305 (99%), Positives = 305/305 (100%), Gaps = 0/305 (0%)

```
Query  38   MKVGKIFFLLNILVVCHFIISCLCRNGQTTRGNLLALKAIEQDLQQKKNRKRNLILYSLG  97
            MKVGKIFFLLNILVVCHFIISCLCRNGQTTRGNLLALKAIEQDLQQKKNRKRNLILYSLG
Sbjct  1    MKVGKIFFLLNILVVCHFIISCLCRNGQTTRGNLLALKAIEQDLQQKKNRKRNLILYSLG  60

Query  98   SAALIAALVVTGIGLNMYMKKKNVDSEVQEIIDEKDEKVKEKPAEKKKTTVKIVSKRVPV  157
            SAALIAALVVTGIGLNMYMKKKNVDSEVQEIIDEKDEKVKEKPAEKKKTTVKIVSKRVPV
Sbjct  61   SAALIAALVVTGIGLNMYMKKKNVDSEVQEIIDEKDEKVKEKPAEKKKTTVKIVSKRVPV  120

Query  158  KSKSSNGKSKARTVNSEVSPKLDDEKKEDLLKFNDNDLLLAAESLKELNPKYDENTQGND  217
            KSKSSNGKSKARTVNSEVSPKLDDEKKEDLLKFNDNDLLLAAESLKELNPKYDENTQGND
Sbjct  121  KSKSSNGKSKARTVNSEVSPKLDDEKKEDLLKFNDNDLLLAAESLKELNPKYDENTQGND  180

Query  218  SFKNINEPRKLASFSLYDALADASEQNKNKDAESSTGQIPTPTESSHGISDGKKDTSTND  277
            SFKNINEPRKLASFSLYDALADASEQNKNKDAESSTGQIPTPTESSHGISDGKKDTSTND
Sbjct  181  SFKNINEPRKLASFSLYDALADASEQNKNKDAESSTGQIPTPTESSHGISDGKKDTSTND  240
```

```
Query  278  MDPLNPYGSSKRNSSENKPTSESKGTTPESNFDSKTPEIKEINEPIIVPSYYPTTGPNPN  337
            MDPLNPYGSSKRNSSE+KPTSESKGTTPESNFDSKTPEIKEINEPIIVPSYYPTTGPNPN
Sbjct  241  MDPLNPYGSSKRNSSEDKPTSESKGTTPESNFDSKTPEIKEINEPIIVPSYYPTTGPNPN  300

Query  338  THGPP  342
            THGPP
Sbjct  301  THGPP  305
```



Annotation: early transcribed membrane protein 10.2 (ETRAMP10.2)

PlasmoDB comments: Integral parasitophorous vacuole membrane protein (Spielmann et al., 2003; Spielmann and Beck, 2000; Birago et al., 2003).

7. PF3D7_1233600 - PFL1620w

Bait: Putative Calmodulin Binding Site

In Frame? No

Sequence:

DGNNNNIINRCNNNNYYYDNMKHVDEKGGEGEGEDSEECQIKESYKKMSECNNKENIIFDSINVLRKNNIKRLKNY
MCKNKNCYIYYDDNNNKKKKNKKNVENQEKEFYVLNKIFVHNFINCINNINVNEDKCFQKVRSTILNRLKEMYSGN
YDCKNNNSNNEFIELAKKKQEDLLKSMKEQQSKFSHFLEEEYSSEENDSLPNGGTEDFEDVDFVDDASSYLDSNSNN
NSDGH

The full insert was not sequenced. The underlined sequence is the ones found in the BLAST search.

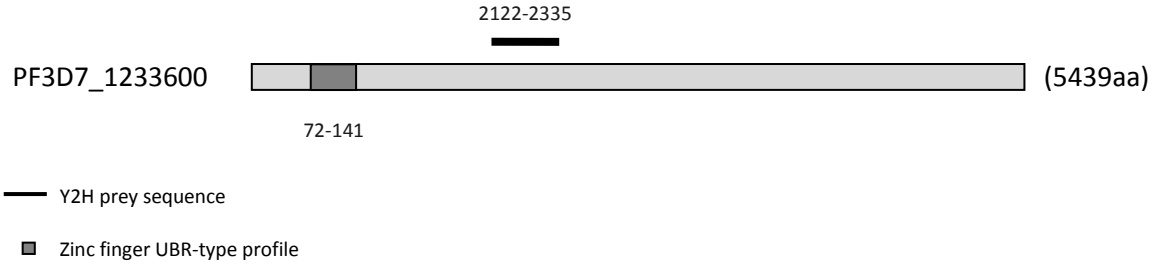
BLAST:

	Score	E
PF3D7_1233600 | organism = Plasmodium_falciparum_3D7 |	313	3e-97
PF3D7_1019300 | organism = Plasmodium_falciparum_3D7 |	29.3	0.87

>PF3D7_1233600

Score = 313 bits (803), Expect = 3e-97, Method: Compositional matrix adjust. Identities = 214/215 (99%), Positives = 215/215 (100%), Gaps = 0/215 (0%)

```
Query  21    MKHVDEKGGEGEGEDSEECQIKESYKKMSECNNKENIIFDSINVLRKNNIKRLKNYMCKN  80
             MKHVDEKGGEGEGEDSEECQIKESYKKMSECNNKENIIFDSINVLRKNNIKRLKNYMCKN
Sbjct  2122  MKHVDEKGGEGEGEDSEECQIKESYKKMSECNNKENIIFDSINVLRKNNIKRLKNYMCKN  2181

Query  81    KNCYIYYDDNNNKKKKNKKNVENQEKEFYVLNKIFVHNFINCINNINVNEDKCFQKVRST  140
             KNCYIYYDDNNNKKKKNKKNVENQEKEFYVLNKIFVHNFINCINNINVNEDKCFQKVRST
Sbjct  2182  KNCYIYYDDNNNKKKKNKKNVENQEKEFYVLNKIFVHNFINCINNINVNEDKCFQKVRST  2241

Query  141   ILNRLKEMYSGNYDCKNNNSNNEFIELAKKKQEDLLKSMKEQQSKFSHFLEEEYSSEEND  200
             ILNRLKEMYSGNYDCKNNNSNNEFIELAKKKQEDLLKSMKEQQSKFSHFLEEEYSSEEND
Sbjct  2242  ILNRLKEMYSGNYDCKNNNSNNEFIELAKKKQEDLLKSMKEQQSKFSHFLEEEYSSEEND  2301

Query  201   SLPNGGTEDFEDVDFVDDASSYLDSNSNNNSDGH  234
             SLPNGGTEDFEDVDFVDDASSYLDSNSNNNSDGH
Sbjct  2302  SLPNGGTEDFEDVDFVDDASSYLDSNSNNNSDGH  2335
```



Annotation: Asparagine and aspartate rich protein 1 (AARP1)

Molecular function prediction: ubiquitin-protein ligase activity, protein binding, zinc ion binding.

8. PF3D7_1324800 - PF13_0140

Bait: Putative Calmodulin Binding Site

In Frame? No

Sequence:

GLRATYNAFTL*TIFFFFFLRGSTIFT*TVHNFKLKKNKMEKNQNDKSNKNDIIHMNDKSGNYDKNNINNFIDKNDEHDMSDILHKINNEEKKYEEIKSYSECLELLYKTHALKLGLDNPKKLNESFGHPCDKYKTIHIAGTNGKGSVCYKIYTCLKIKKFKVGLFSSPHIFSLRERIIVNDEPISEKELIHLVNEVLNKAKKLYINPSFFEIITLVAFLHFLNKKVDYAIIETGIGGRLDATNILTKPEVIVITSIGYDHLNILGDNLPIICNEKIGIFKKDANVVIGPSVAIYKNVFDKAKELNCTIHTVVPEPRGERFNEENSRIALRTLEILNISIDYFLKSIIPIKPPLRI

The full insert was not sequenced. The underlined sequence is the ones found in the BLAST search.

BLAST:

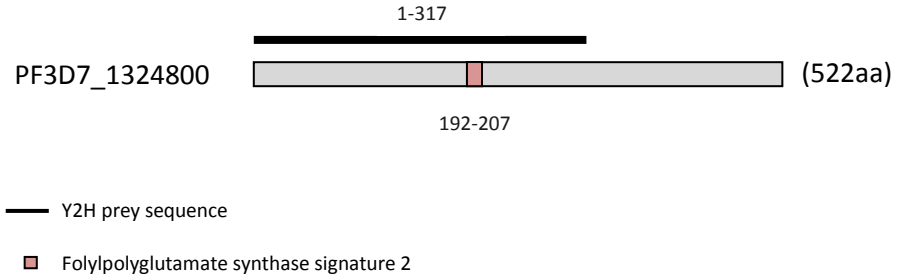
		Score	E
PF3D7_1324800	organism = Plasmodium_falciparum_3D7	644	0.0
PF3D7_0910700	organism = Plasmodium_falciparum_3D7	34.7	0.031
PF3D7_0215600	organism = Plasmodium_falciparum_3D7	33.9	0.054
PF3D7_1107300	organism = Plasmodium_falciparum_3D7	30.8	0.71
PF3D7_1219000	organism = Plasmodium_falciparum_3D7	30.8	0.62
PF3D7_1327300	organism = Plasmodium_falciparum_3D7	30.0	0.93
PF3D7_0934100	organism = Plasmodium_falciparum_3D7	28.9	2.1
PF3D7_1035000	organism = Plasmodium_falciparum_3D7	28.5	3.4
PF3D7_0619000.1	organism = Plasmodium_falciparum_3D7	28.1	4.4
PF3D7_0619000.2	organism = Plasmodium_falciparum_3D7	28.1	4.5
PF3D7_1324000	organism = Plasmodium_falciparum_3D7	26.9	9.9
PF3D7_1352000	organism = Plasmodium_falciparum_3D7	26.9	9.4

>PF3D7_1324800

Score = 644 bits (1661), Expect = 0.0, Method: Compositional matrix adjust. Identities = 325/340 (96%), Positives = 329/340 (97%), Gaps = 1/340 (0%)

```
Query  40   MEKNQNDKSNKNDIIHMNDKSGNYDKNNINNFIDKNDEHDMSDILHKINNEEKKYEEIKS  99
            MEKNQNDKSNKNDIIHMNDKSGNYDKNNINNFIDKNDEHDMSDILHKINNEEKKYEEIKS
Sbjct  1    MEKNQNDKSNKNDIIHMNDKSGNYDKNNINNFIDKNDEHDMSDILHKINNEEKKYEEIKS  60

Query  100  YSECLELLYKTHALKLGLDNPKKLNESFGHPCDKYKTIHIAGTNGKGSVCYKIYTCLKIK  159
            YSECLELLYKTHALKLGLDNPKKLNESFGHPCDKYKTIHIAGTNGKGSVCYKIYTCLKIK
Sbjct  61   YSECLELLYKTHALKLGLDNPKKLNESFGHPCDKYKTIHIAGTNGKGSVCYKIYTCLKIK  120

Query  160  KFKVGLFSSPHIFSLRERIIVNDEPISEKELIHLVNEVLNKAKKLYINPSFFEIITLVAF  219
            KFKVGLFSSPHIFSLRERIIVNDEPISEKELIHLVNEVLNKAKKLYINPSFFEIITLVAF
Sbjct  121  KFKVGLFSSPHIFSLRERIIVNDEPISEKELIHLVNEVLNKAKKLYINPSFFEIITLVAF  180
```

```
Query  220  LHFLNKKVDYAIIETGIGGRLDATNILTKPEVIVITSIGYDHLNILGDNLPIICNEKIGI  279
            LHFLNKKVDYAIIETGIGGRLDATNILTKPEVIVITSIGYDHLNILGDNLPIICNEKIGI
Sbjct  181  LHFLNKKVDYAIIETGIGGRLDATNILTKPEVIVITSIGYDHLNILGDNLPIICNEKIGI  240

Query  280  FKKDANVVIGPSVAIYKNVFDKAKELNCTIHTVVPEPRGERFNEENSRIALRTLEILNIS  339
            FKKDANVVIGPSVAIYKNVFDKAKELNCTIHTVVPEPRGER+NEENSRIALRTLEILNIS
Sbjct  241  FKKDANVVIGPSVAIYKNVFDKAKELNCTIHTVVPEPRGERYNEENSRIALRTLEILNIS  300

Query  340  IDYFLKSIIPIKPPLRI  356
            IDYFLKSIIPIKPPLRI
Sbjct  301  IDYFLKSIIPIKPPLRI  317
```



Annotation: dihydrofolate synthase/folylpolyglutamate synthase (DHFS-FPGS)

9. PF3D7_0220000 - PF02_0187 - PFB0915w

Bait: PfCRT N-term

In Frame? No

Sequence:

GEIFDNVKRIHYKLLTSPFLRIETNLKIQSEQKVDLNANEGSSIFYNIKKMK

Poor sequence quality. The full insert was not sequenced. The underlined sequence is the one found in the BLAST search.

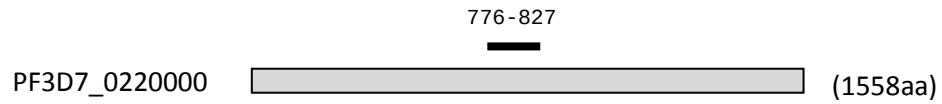
BLAST:

	Score	E
PF3D7_0220000 | organism = Plasmodium_falciparum_3D7|	58.5	8e-12
PF3D7_1028700 | organism = Plasmodium_falciparum_3D7|	27.7	0.11
PF3D7_1110500 | organism = Plasmodium_falciparum_3D7|	27.3	0.19
PF3D7_1208200 | organism = Plasmodium_falciparum_3D7|	23.1	3.6
PF3D7_0820000 | organism = Plasmodium_falciparum_3D7|	22.3	8.4

>PF3D7_0220000

Score = 58.5 bits (140), Expect = 8e-12, Method: Compositional matrix adjust. Identities = 32/52 (62%), Positives = 36/52 (69%), Gaps = 0/52 (0%)

```
Query  1    GEIFDNVKRIHYKLLTSPFLRIETNLKIQSEQKVDLNANEGSSIFYNIKKMK  52
            EIFDNVK I   LLT  F  IET++ IQSE+KVDLN N  SSI  NI+ MK
Sbjct  776  SEIFDNVKGIQENLLTGMFRSIETSIVIQSEEKVDLNENVVSSILDNIENMK  827
```



No predicted domains by Prosite.

Annotation: Liver stage antigen 3 (LSA3)

10. PF3D7_0406500 - PFD0320c

Bait: PfCRT C-term

In Frame? Yes

Sequence:

DKMTLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEIKNFRNDKITLEKEIKNFRNDKMTLEKEIKNFSNDKITLEKEIQNIR
NEKITIEKEIQNISNDKMTLEKEIQNIRNDKIVFEEEKKKFLDNKETITYEIKKSILIDNLCVKEKQKFLNIKNEEIKLDLDKL
NIKDEREKLDKDKIEMENEKESFCKEKKAYELKKEDLELDVIIVDIQKKMIKENFEKIEDEKRDFRIEILKPIERLNRVTNY
LYYKKALKKYNKHGKEQNLKYNKYTNKNKDTEEENSNSDIYGDMFLKYSSNVNKSNKDTSKDVINKTI

The full insert was not sequenced. The underlined sequence is the one found in the BLAST search.

BLAST:

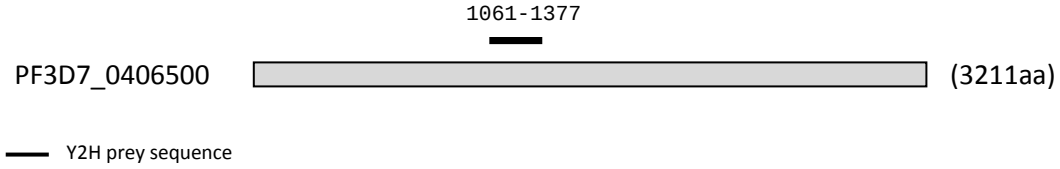
	Score	E
PF3D7_0406500 | organism = Plasmodium_falciparum_3D7 |	573	0.0
PF3D7_0423600 | organism = Plasmodium_falciparum_3D7 |	38.9	0.001
PF3D7_1252400 | organism = Plasmodium_falciparum_3D7 |	36.2	0.011
PF3D7_0322800 | organism = Plasmodium_falciparum_3D7 |	31.2	0.21
PF3D7_0317300 | organism = Plasmodium_falciparum_3D7 |	28.5	2.8
PF3D7_1021800 | organism = Plasmodium_falciparum_3D7 |	28.5	2.8
PF3D7_0710200 | organism = Plasmodium_falciparum_3D7 |	28.1	3.4
PF3D7_1126700 | organism = Plasmodium_falciparum_3D7 |	28.1	3.5
PF3D7_0204300 | organism = Plasmodium_falciparum_3D7 |	27.3	5.9
PF3D7_1117200 | organism = Plasmodium_falciparum_3D7 |	26.9	8.3

>PF3D7_0406500

Score = 573 bits (1478), Expect = 0.0, Method: Compositional matrix adjust. Identities = 312/318 (98%), Positives = 314/318 (99%), Gaps = 0/318 (0%)

```
Query  1     DKMTLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEIKNFRNDKITLEKEIKNFRNDKM  59
             DKMTLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEI+N  NDK+TLEKEIKNFRNDKM
Sbjct  1061  DKMTLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEIQNISNDKMTLEKEIKNFRNDKM  1119

Query  60    TLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEIQNISNDKMTLEKEIQNIRNDKIVFEE  119
             TLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEIQNISNDKMTLEKEIQNI NDKIVFEE
Sbjct  1120  TLEKEIKNFSNDKITLEKEIQNIRNEKITIEKEIQNISNDKMTLEKEIQNISNDKIVFEE  1179

Query  120   EKKKFLDNKETITYEIKKSILIDNLCVKEKQKFLNIKNEEIKLDLDKLNIKDEREKLDKD  179
             EKKKFLDNKETITYEIKKSILIDNLCVKEKQKFLNIKNEEIKLDLDKLNIKDEREKLDKD
Sbjct  1180  EKKKFLDNKETITYEIKKSILIDNLCVKEKQKFLNIKNEEIKLDLDKLNIKDEREKLDKD  1239
```

```
Query  180   KIEMENEKESFCKEKKAYELKKEDLELDVIIVDIQKKMIKENFEKIEDEKRDFRIEILKP  239
             KIEMENEKESFCKEKKAYELKKEDLELDVIIVDIQKKMIKENFEKIEDEKRDFRIEILKP
Sbjct  1240  KIEMENEKESFCKEKKAYELKKEDLELDVIIVDIQKKMIKENFEKIEDEKRDFRIEILKP  1299

Query  240   IERLNRVTNYLYYKKALKKYNKHGKEQNLKYNKYTNKNKDTEEENSNSDIYGDMFLKYSS  299
             IERLNRVTNYLYYKKALKKYNKHGKEQNLKYNKYTNKNKDTEEENSNSDIYGDMFLKYSS
Sbjct  1300  IERLNRVTNYLYYKKALKKYNKHGKEQNLKYNKYTNKNKDTEEENSNSDIYGDMFLKYSS  1359

Query  300   NVNKSNKDTSKDVINKTI  317
             NVNKSNKDTSKDVINKTI
Sbjct  1360  NVNKSNKDTSKDVINKTI  1377
```



No predicted domains by Prosite.

Annotation: Conserved Plasmodium protein, unknown function