Factors contributing to artemisinin resistance

in *Plasmodium falciparum* parasites

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Summary

Plasmodium falciparum is a unicellular protozoan parasite transmitted by the bite of an infected female Anopheles mosquito. Of the five human-infecting *Plasmodium* species, *P. falciparum* is the most virulent. Although malaria is preventable and curable, in 2019 almost half of the world´s population was still at risk to suffer from malaria with the highest risk seen in sub-Saharan Africa. To date, the most effective treatment for uncomplicated *P. falciparum* malaria recommended by the WHO is the artemisinin-based combination therapy (ACT), combining a short-acting artemisinin derivative (ART) with a longer-acting partner drug. In 2008, artemisinin (ART) resistant parasites were reported for the first time. This resistance was detected in Cambodia and manifested as a delayed parasite clearance after ART treatment in malaria patients. Thereafter, ART resistance spread across the entire Southeast Asian subcontinent. For a long time, the African continent seemed to be unaffected by ART resistant strains, but in 2020 low prevalent ART resistant *P. falciparum* strains were also reported for Rwanda, Tanzania, and Uganda. Since Africa carries the largest malaria burden, affecting mostly children under the age of five and pregnant women, resistance to the most effective drug would be devastating.

In 2014, mutations in the *Pf*Kelch13 protein propeller domain were identified as a molecular marker for ART resistance and demonstrated to be responsible for a reduced susceptibility of young ring stage parasites to ART. The resistance can be measured *in vitro* using a ring-stage survival assay (RSA). Several studies revealed that besides *Pf*Kelch13, mutations in other genes can cause ART resistance, suggesting that also *Pf*Kelch13-independent resistance mechanisms exist. Using a novel dimerization-induced quantitative BioID (DiQ-BioID) approach, a new cellular compartment, where *Pf*Kelch13 is located, was defined. In this thesis proteins of this compartment were analyzed. Upon partial inactivation of either *Pf*Kelch13 itself or of essential Kelch13 compartment members (*Pf*AP-2μ, *Pf*Eps15, *Pf*UBP1, *Pf*KIC7), parasites became resistant to ART, indicating that they are involved of ART resistance in *P. falciparum*. In addition, two further hits from the DiQ-BioID, *Pf*MCA2 and *Pf*MyosinF, were analyzed in this work. Co-localization experiments confirmed that *Pf*MCA2 is indeed part of the Kelch13 compartment but is also localized to additional foci in the periphery of the parasite cell. A mutation, leading to a premature stop codon at amino acid position 1344 of *Pf*MCA2 rendered parasites resistant to ART when it was introduced in 3D7 parasites, confirming a role of *Pf*MCA2 in ART resistance. While the SNP, leading to this pre-mature stop, was detected in African *Plasmodium* strains in patient field samples, this SNP only seemed to co-occur with another mutation in the same codon, resulting in an

amino acid change rather than a stop. This indicated that this SNP is not relevant for ART resistance in the field.

Performing co-localization experiments with *Pf*MyosinF demonstrated that this protein is also part of the Kelch13 compartment, although fully overlapping signals were only observed for ~14% of the *Pf*MyosinF foci and overall, *Pf*MyosinF was mostly found in close association to *Pf*Kelch13 positive structures rather than fully overlapping. Myosins are part of the actomyosin motor to generate movement of cellular components. Inhibition of actin-polymerization by Cytochalasin D treatment caused the dissociation of *Pf*MyosinF and *Pf*Kelch13 positive structures in *P. falciparum* in trophozoite stage parasites, indicating that the association of both proteins - or the structures they are contained in - is actin-dependent. Furthermore, although mislocalization of *Pf*MyosinF by knock sideways was only partially successful, this nevertheless significantly reduced parasite growth, suggesting that *Pf*MyosinF is important for *Plasmodium* blood stage development. However, this partial inactivation of *Pf*MyosinF did not render parasites resistant to ART as determined by RSA. Additionally, it was observed in this work that *Pf*MyosinF is located in proximity of phosphatidylinositol-3-phosphate positive structures in the parasite cell, suggesting it is proximal to endosomal structures. Finally, partial inactivation reduced uptake of hemoglobin into the parasite's food vacuole. Overall, these findings indicate a role of *Pf*MyosinF in hemoglobin endocytosis, similar to Kelch13 compartment proteins.

As this and previous work indicated that Kelch13 compartment proteins are all involved in the same pathway that can influence the susceptibility of the parasite to ART, it was in a next step tested whether non-synonymous single nucleotide polymorphisms (SNPs) in these genes could also mediate ART resistance in the field. As few *Pf*Kelch13 mutations were so far reported from Africa, one focus was set on SNPs in African isolates to assess if they might have gone unnoticed, as most previous studies focused on *pfkelch13*. For this part of the work, different approaches were used. One approach was based on the simultaneous introduction of multiple non-synonymous mutations in one genomic locus of the 3D7 laboratory isolate, using the selection-linked integration system (SLI), creating mutation pools. In total, 125 mutations in eight different genes (*pfkic1* (PF3D7_0606000), *pfkic2* (PF3D7_1227700), *pfkic4* (PF3D7_1246300), *pfkic5* (PF3D7_1138700), *pfkic7* (PF3D7_0813000), *pfkic9* (PF3D7_1442400), *pfmyosinF* (PF3D7_1329100), *pfubp1* (PF3D7_0104300)) were tested with this method. These experiments showed that none of the included mutations changed parasite susceptibility to ART. In a second approach, non-synonymous mutations that either were mentioned in the literature or were detected by sequencing the DNA of a potentially resistant *P. falciparum* field isolate obtained during the 'Fever without Source' study in Ghana, were tested individually. In total, 11 non-

synonymous mutations in five different genes were tested, including mutations in *pfkelch13* itself. This revealed that two mutations of *pfkelch13* (leading to amino acid change V520A and V589I) and one mutation of *pfubp1* (leading to amino acid change R3138H) conferred a reduced susceptibility to ART upon introduction into the 3D7 parasite genome, while one mutation found in *pfap-2α* (leading to amino acid change H817P) decreased ART susceptibility but did not reach the threshold of 1% survival in RSA to define it as a resistance mutation. All of the other tested mutations, including in *pfeps15* and *pfmyosinF,* did not result in ART resistance.

Additionally, confocal microscopy of ART resistant parasites was performed to follow up the growth of the parasites after an ART pulse in an RSA. This work indicated that either ART treatment delayed parasite development after the drug pulse, or that a subset of more slowly growing parasites survived. Conducting consecutive RSA with the already resistant C580Y mutant parasites rendered these parasites even more resistant (K13$^{C580Y\_29th}$). When the *Pf*Kelch13 protein level of these highly ART resistant K13$^{C580Y\_29th}$ parasites was compared to *Pf*Kelch13 wild type, unselected resistant C580Y parasites and the mildly resistant V520A parasites, the K13$^{C580Y\_29th}$ parasites displayed the lowest protein amount. This correlated inversely with the parasite survival rate in RSA. Competitive growth assays performed with different *Pf*Kelch13 mutant lines revealed that the ART resistant C580Y mutant had the highest fitness cost, while the moderate resistant V520A mutant had a similar fitness compared to *Pf*Kelch13 wild type parasites. These results suggest that there is also a correlation between the degree of ART resistance and the level of fitness of the parasite. Hence, lower *Pf*Kelch13 levels result in lower fitness but higher resistance.

Altogether, this work provided further evidence that *Pf*Kelch13 and its compartment members are important for a pathway relevant for ART resistance. Mutations, either detected in *Pf*Kelch13 or the compartment members, reduced parasite susceptibility to the drug. The mutation tested in *Pf*UBP1 demonstrated that resistance exists in the field that is independent of changes in *Pf*Kelch13. This may indicate that further diagnostic markers for ART resistance exist in the field. Furthermore, this work shows that there are mutations in *pfkelch13* found in African parasite isolates that influence *Pf*Kelch13 protein levels as well as fitness of the parasite but to a more moderate level than the most prevalent mutation (C580Y) found in Southeast Asia. This data could help to understand the reason for the unequal spread of ART resistance in different areas of the world.

Zusammenfassung

Plasmodium falciparum ist ein einzelliger, protozoischer Parasit, welcher durch den Stich einer infizierten weiblichen *Anopheles* Mücke übertragen wird. Unter insgesamt fünf humanpathogenen Plasmodien-Erregern ist *Plasmodium falciparum* die virulenteste Art. Obwohl die Krankheit Malaria durch vorbeugende Maßnahmen gut zu kontrollieren ist und effektive Medikamente zur Behandlung einer Infektion erhältlich sind, stellt die parasitäre Erkrankung auch im Jahr 2019 eines der größten Gesundheitsprobleme weltweit dar. Ungefähr die Hälfte der Gesamtbevölkerung der Erde ist dem Risiko einer Infektion ausgesetzt, wobei Afrika südlich der Sahara am stärksten betroffen ist. Das wirksamste, von der WHO empfohlene Medikament zur Behandlung einer Malariaerkrankung ist die Artemisinin-basierte Kombinationstherapie (ACT). Hierbei werden ein kurzlebiges Artemisinin-Derivat und ein langfristig wirkendes Partnermedikament kombiniert. Erste Resistenzen gegenüber Artemisinin wurden im Jahr 2008 in Kambodscha beobachtet. Diese äußerten sich in einer verlangsamten Eliminierung des Parasiten im Blut. In den darauffolgenden Jahren verbreitete sich die Resistenz über den gesamten südostasiatischen Subkontinent. Für lange Zeit schien der afrikanische Kontinent von Artemisinin-Resistenz verschont zu bleiben, allerdings wurden auch hier im Jahr 2020 die ersten resistenten Plasmodien-Stämme in Ruanda, Tansania und Uganda detektiert. Da Afrika am stärksten von der Infektionskrankheit betroffen ist, und dort in den meisten Fällen Kinder im Alter bis fünf Jahre oder Schwangere schwer an Malaria erkranken, würde eine Medikamenten-Resistenz verheerende Auswirkungen haben.

Im Jahr 2014 konnte eine Mutation in der Propeller-Domäne des *Pf*Kelch13 Proteins als molekularer Marker der Artemisinin Resistenz identifiziert werden und für eine reduzierte Sensitivität junger Ringstadien gegenüber Artemisinin verantwortlich gemacht werden. Diese Resistenz kann anhand eines sogenannten „ring-stage survival assays" (RSA) *in vitro* gemessen werden. Einige Studien deuten darauf hin, dass es neben der durch *Pf*Kelch13 vermittelten Resistenz eine *Pf*Kelch13 unabhängige Form der Artemisinin-Resistenz gibt. Anhand einer neuentwickelten Methode, die auf einer quantitativen BioID basiert (DiQ-BioID), wurden verschieden Interaktionspartner des *Pf*Kelch13 Proteins bestimmt, die ein bis dahin unbekanntes Zellkompartiment, in welchem *Pf*Kelch13 lokalisiert ist, definieren. In der vorliegenden Arbeit wurden Proteine dieses Kompartiments analysiert. Durch die partielle Inhibierung des *Pf*Kelch13 Proteins, aber auch der essenziellen Kompartimentproteine (*Pf*AP-2μ, *Pf*Eps15, *Pf*UBP1, *Pf*KIC7), wurden die Parasiten resistent gegen Artemisinin, was darauf hindeutet, dass sie an einem Prozess beteiligt sind, der für den Resistenzmechanismus in *P. falciparum* wichtig ist. Zusätzlich wurden in dieser

Arbeit zwei bisher noch nicht untersuchte Kandidaten aus der DiQ-BioID, *Pf*MCA2 und *Pf*MyosinF, genauer funktionell studiert. Co-Lokalisationsexperimente zeigten, dass *Pf*MCA2 tatsächlich Teil des Kelch13 Kompartiments ist, allerdings auch in anderen Foci in der Zellperipherie gefunden werden kann. Eine Mutation, die zu einem vorzeitigen Stopp an der Position 1344 in *Pf*MCA2 führte, resultierte darin, dass die Parasiten resistent gegenüber Artemisinin wurden. Dies bekräftigt, dass die Funktion von *Pf*MCA2 die Artemisininresistenz beeinflusst. Allerdings scheint diese Mutation, welche in afrikanischen Feldisolaten vorhanden ist, nur zusammen mit einer zweiten Mutation im selben Codon aufzutreten, wodurch es zu einem Aminosäureaustausch statt eines Stopps kommt und darauf hindeutet, dass diese Mutation daher für die Resistenz im Feld nicht relevant ist.

Co-Lokalisationsexperimente mit *Pf*MyosinF zeigten, dass dieses Protein ebenfalls Teil des *Pf*Kelch13 Kompartiments ist, obwohl komplett überlappende Signale nur in ~14% der *Pf*MyosinF Foci beobachtet werden konnte und *Pf*MyosinF meist nur in der näheren Umgebung zu *Pf*Kelch13-poitiven Strukturen gefunden werden konnte, aber keine vollständige Überlappung zeigte. Myosine sind Teil des Actomyosinmotors, welcher Bewegung zellulärer Komponenten generiert. Inhibierung der Polymerisation von Aktinfilamenten durch Cytochalasin D führte zur Dissoziation von *Pf*MyosinF- und *Pf*Kelch13-positiven Strukturen in Trophozoitenstadien, was darauf hindeutet, dass die Interaktion beider Proteine (oder der durch diese Proteine definierten Strukturen) Aktin-abhängig ist. Desweiteren konnte anhand von Wachstumsexperimenten gezeigt werden, dass obwohl die Mislokalisation von *Pf*MyosinF durch das knock sideways-System nur partiell möglich war, diese partielle Inaktivierung das Wachstum der Blutstadien des Parasiten signifikant reduzierte. Allerdings resultierte eine partielle Inaktivierung von *Pf*MyosinF nicht in Resistenz der Plasmodien gegenüber Artemisinin, was durch einen RSA getestet wurde. Zusätzlich konnte in dieser Arbeit gezeigt werden, dass *Pf*MyosinF in der Nähe zu Phosphatidylinositol-3-phosphat-positiven Strukturen im Parasiten lokalisiert ist, was vermuten lässt, dass es sich in der Nähe zu endosomalen Strukturen befindet. Schließlich konnte gezeigt werden, dass die teilweise Inaktivierung von *Pf*MyosinF zu einer reduzierten Aufnahme von Hämoglobin in die Fressvakuole des Parasiten führte. Zusammengefasst deuten die Ergebnisse darauf hin, dass *Pf*MyosinF - ähnlich wie andere Kelch13 Kompartimentproteine - eine Rolle während der Endozytose von Hämoglobin spielt.

Da diese und vorherige Arbeiten gezeigt haben, dass die Kelch13 Kompartimentproteine am selben Prozess beteiligt sind, welcher auch die Sensitivität der Parasiten gegenüber Artemisinin beeinflusst, wurde in einem nächsten Schritt getestet, ob verschiedene nicht-synonyme Mutationen in diesen *Pf*Kelch13-Kompartimentproteinen Artemisinin-Resistenz

im Feld vermitteln. Da bisher nur wenige Mutationen in *pfkelch13* in Afrika festgestellt wurden, wurde ein spezieller Fokus auf afrikanische Varianten gerichtet, um zu untersuchen, ob diese übersehen wurden, da sich die meisten Studien bisher nur mit *pfkelch13* Mutationen befasst haben. Für diesen Teil der Arbeit wurden verschiedene Ansätze gewählt. Im ersten Ansatz wurden mehrere verschiedene nicht-synonyme Mutationen gleichzeitig über das SLI-System in einen genomischen Locus des 3D7 Laborstammes integriert und damit sogenannte Mutationspools erzeugt. Insgesamt wurden auf die Weise 125 Mutationen in acht verschiedenen Genen (*pfkic1* (PF3D7_0606000), *pfkic2* (PF3D7_1227700), *pfkic4* (PF3D7_1246300), *pfkic5* (PF3D7_1138700), *pfkic7* (PF3D7_0813000), *pfkic9* (PF3D7_1442400), *pfmyosinF* (PF3D7_1329100), *pfubp1* (PF3D7_0104300)) getestet. Die damit durchgeführten Versuche zeigten, dass keine der eingefügten Mutationen die Toleranz des Parasiten gegenüber Artemisinin verringert hat. Im zweiten Ansatz wurde verschiedenen nicht-synonyme Mutationen, die entweder in der Literatur erwähnt wurden, oder mittels Sequenzierung von Parasiten-DNA eines potenziell resistenten Stammes aus einem Patienten der „Fever without Source"-Studie in Ghana stammten, individuell getestet. Insgesamt wurden 11 verschiedene Mutationen in fünf Genen getestet, wobei auch Mutationen in *pfkelch13* selbst miteinbezogen wurden. Dabei konnten zwei *pfkelch13* Mutationen (resultieren in den folgenden Aminosäureaustauschen: V520A und V589I) und eine Mutation in *pfubp1* (resultiert im Aminosäureaustausch R3138H) identifiziert werden, die zu einer reduzierten Sensitivität gegenüber Artemisinin führten, wenn sie in 3D7 *P. falciparum* Genom eingebracht wurden, während eine Mutation in *pfap-2α* (resultiert im Aminosäureaustausch H817P) ebenfalls die Sensitivität gegenüber ART reduzierte, jedoch nicht den Grenzwert von 1% Überlebensrate im RSA überschritt (der Grenzwert um als Resistenz-vermittelnde Mutation bezeichnet zu werden). Keine der anderen getesteten Mutationen, einschließlich derer in den Kandidaten *pfeps15* und *pfmyosinF,* führten zu einer erhöhten Toleranz gegenüber Artemisinin.

Zusätzlich wurde ein RSA mit Artemisinin resistente Parasiten durchgeführt und die Entwicklung der Parasiten nach dem Artemisinin Puls mittels Konfokalmikroskopie nachverfolgt. Anhand der gewonnenen Daten konnte gezeigt werden, dass sich das Wachstum der Parasiten nach Artemisinin-Gabe verlangsamte bzw., dass ein generell verlangsamtes Wachstum einer Subpopulation an Parasiten ermöglichte, zu überleben. Desweiteren erreichten Artemisinin-resistente C580Y Parasiten ein noch höheres Resistenzlevel, wenn sie in einem konsekutiv durchgeführten RSA wiederholt mit dem Medikament behandelt wurden (K13$^{C580Y\_29th}$). Die quantitative Bestimmung der *Pf*Kelch13-Menge dieser hochresistenten K13$^{C580Y\_29th}$ Parasiten ergab, dass sie im Vergleich zu *Pf*Kelch13-Wildtyp Parasiten, unselektierten C580Y Parasiten und den moderat

resistenten V520A Mutanten die geringste Proteinmenge exprimierten. Dabei korrelierten die *Pf*Kelch13-Proteinmenge und das Artemisinin-Resistenzlevel im RSA invers miteinander. Kompetitive Wachstumsexperimente mit den verschiedenen *Pf*Kelch13-Mutanten zeigten, dass die Artemisinin resistente C580Y Mutation die höchsten Fitnesskosten verursachte, während die moderat-resistente V520A Mutante eine ähnliche Fitness aufwies wie die *Pf*Kelch13 Wildtyp-Parasiten. Diese Ergebnisse deuteten darauf hin, dass auch das Artemisininresistenz-Level und die Fitness der Parasiten miteinander korrelieren. Demnach führt ein geringeres *Pf*Kelch13-Level zwar zu einer geringeren Fitness, dafür aber zu einer höheren Resistenz gegenüber Artemisinin.

Zusammengefasst konnten mit dieser Arbeit weitere Beweise dafür geliefert werden, dass *Pf*Kelch13 und seine Kompartimentproteine wichtige Regulatoren eines Signalweges darstellen, der für die Artemisininresistenz wichtig ist. Mutationen in *Pf*Kelch13 oder Kelch13 Kompartimentproteinen resultierten in einer erhöhten Toleranz gegenüber Artemisinin. Dabei zeigt die Mutation in *Pf*UBP1, dass Resistenz unabhängig von *Pf*Kelch13 im Feld existiert. Zusätzlich zeigt diese Arbeit, dass in Afrika vorkommende Mutationen in *pfkelch13* sowohl das *Pf*Kelch13 Proteinlevel als auch die Fitness der Parasiten auf eine moderatere Weise reduzieren, wenn dies mit der am häufigsten in Südostasien vorkommenden C580Y Variante verglichen wird. Diese Daten können dazu beitragen, die Ursachen der ungleichmäßigen Verbreitung von Artemisininresistenz in den verschiedenen Malaria-endemischen Regionen der Erde zu verstehen.

Table of contents

List of Figures

XVII

List of Tables

Abbreviations

aa	amino acid
ACT	artemisinin combination therapy
AMA1	apical membrane antigen 1
AQ	amodiaquine
ART	artemisinin and its derivatives
AS	artesunate
BSV	blood-stage vaccine
CSP	circumsporozoite protein
DAPI	4´,6-Diamidin-2-phenylindol
DHA	dihydroartemisinin
DIC	differential interference contrast
DiQ-BioID	dimerization-induced quantitative BioID
DNA	deoxyribonucleic acid
DP	dihydroartemisinin-piperaquine
DV	digestive vacuole
EBL	erythrocyte binding-like
EMA	European Medicine Agency
EPM	erythrocyte plasma membrane
ER	endoplasmic reticulum
EXP2	exported protein 2
FV	food vacuole
GDV1	gametocyte development 1
GFP	green fluorescent protein
GMS	Greater Mekong Subregion
GWAS	genome wide association study
h	hour
HA	hemagglutinin
HDA2	histone deacetylase 2
HIV	human immunodeficiency viruses
HP1	heterochromatin protein 1
HRP	horseradish peroxidase
HSP101	heat shock protein 101
IFA	immunofluorescence assay
IMC	inner membrane complex

IRS	indoor residual spray
ITN	insecticide treated net
K13	Kelch13 protein
KAHRP	knob associated histidine-rich protein
kDa	kilodaltons
KIC	Kelch13 interaction candidates
KLHL	Kelch-like gene family
LLINs	long lasting insecticidal nets
LysoPC	Lysophosphatidylcholine
MAHRP1	Maurer`s cleft protein 1
MC	Maurer`s cleft
MQ	mefloquine
MSRP6	merozoite surface related protein 6
MW	molecular weight
NLS	nuclear localization signal
nM	nanomolar
PCR	polymerase chain reaction
PEV	pre-erythrocytic vaccine
PEXEL	*Plasmodium* export element
*Pf*AP-2μ	*P. falciparum* adaptor protein complex subunit mu
*Pf*CRT	*P. falciparum* chloroquine resistance transporter
*Pf*DPAP1	*P. falciparum* dipeptidyl aminopeptidase 1
*Pf*EMP1	*P. falciparum* erythrocyte membrane protein 1
*Pf*MCA2	*P. falciparum* metacaspase 2
*Pf*MDR1	*P. falciparum* multidrug resistance protein 1
*Pf*PI3K	*P. falciparum* phosphatidylinositol 3-kinase
*Pf*Rh	*P. falciparum* reticulocyte binding-like proteins
*Pf*RH5	*P. falciparum* reticulocyte binding protein homologue
*Pf*UBP1	*P. falciparum* ubiquitin carboxyl-terminal hydrolase 1
*Pf*VP1	*P. falciparum* V-type H(+)-translocating pyrophosphatase
PI3P	phosphatidylinositol 3-phosphate
PNEP	PEXEL negative exported protein
PPM	parasite plasma membrane
PTEX	*Plasmodium* translocon of exported proteins
PTP1	protein tyrosine phosphatase
PV	parasitophorous vacuole

PVM	parasitophorous vacuole membrane
RBC	red blood cell
RESA	ring-infected erythrocyte surface antigen
REX1	ring-exported protein 1
REX2	ring-exported protein 2
RFP	red fluorescent protein
RIMA	ring membrane antigen
RNA	ribonucleic acid
RON	rhoptry neck protein
RSA	ring-stage survival assay
SBP1	skeleton-binding protein 1
SEA	Southeast Asia
SLI	selection linked integration
SNP	single nucleotide polymorphism
SP	sulphadoxine-pyrimethamine
STEVOR	subtelomeric variant open reading frame
SUB	subtilisin-like serine protease
TBV	transmission blocking vaccine
TGD	targeted gene disruption
TRX2	thioredoxin 2
TSA	trophozoite-stage survival assay
TVN	tubovesicular network
UN	untreated nets
UPR	unfolded protein response
WHO	World Health Organization
WSV	whole sporozoite vaccine
wt	wild type

1 Introduction

1.1 Malaria – Key facts

1.1.1 Discovery and taxonomic classification of *Plasmodium* parasites

The French Alphonse Charles Laveran was garrisoned in 1878 as military doctor in Algeria where many soldiers were suffering from malaria, but at that time the origin of the disease was unknown. Laveran was very ambitious and persistently worked on elucidating the causal agent of this febrile disease and finally was the first to achieve the microscopical description of the protozoan parasite in the blood of infected soldiers (Alphonse Charles Laveran, 1881).

The organisms he described are obligate intracellular parasites whose motile invasive stages exhibit an evolutionary unique apical complex (Morrison, 2008) that serves as eponym of the phylum Apicomplexa (also known as Sporozoa). Together with dinoflagellates and ciliates, the Apicomplexans belong to the taxonomic group of the Alveolata (Gould *et al.*, 2008; Moore *et al.*, 2008; Yoon *et al.*, 2008). All Apicomplexans are characterized by a parasitic lifestyle and a wide range of animal species can be infected by these unicellular protists. The phylum itself is subclassified in four traditional groups, namely coccidians, gregarines, haemosporidian and piroplasmides (Adl *et al.*, 2005; Morrison, 2008). Covering more than 200 species, the genus *Plasmodium* belongs to the Apicomplexan subgroup of Haemosporidia (Danilewsky, 1885). Out of those five species, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*, are known to infect humans, while the other plasmodial species are capable of infecting birds, reptiles, or mammals (Martine Zilversmit & Daniel L. Hartl, 2014). Among the five human infecting species, *P. falciparum* is the most virulent.

1.1.2 Epidemiology

Although malaria is preventable and curable, still almost half of the world´s population is at risk of this disease with 87 countries being malaria endemic. In 2019, the WHO reported an estimated 229 million clinical malaria episodes and 409 000 deaths, with the WHO African Region having the globally highest disease burden of 94% of reported deaths (Figure 1). In

general, the overall malaria burden declined since the year 2000 and several countries were declared as permanently malaria-free between 2000 and 2019 (WHO, 2020b).
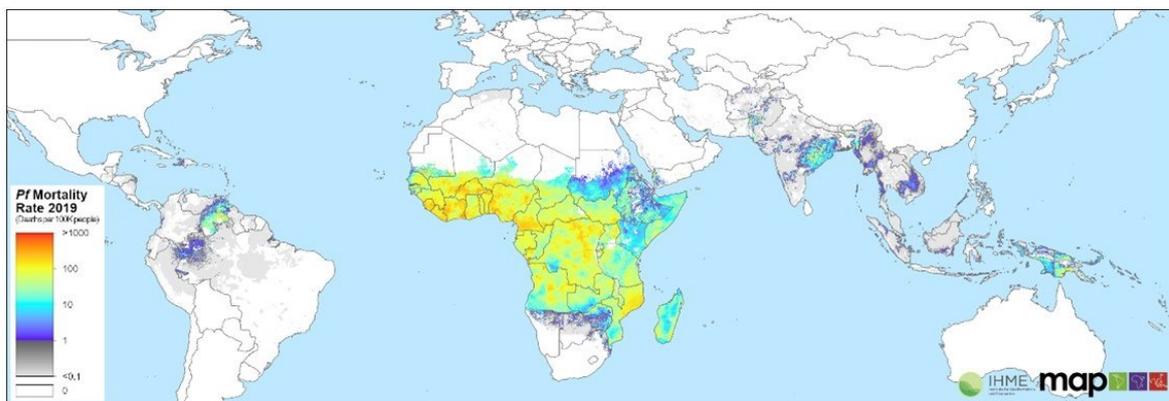
Figure 1) Global *P. falciparum* death rate in 2019. Death incidence due to malaria per 100K people of all ages is indicated in color code. Red color depicts countries with a high mortality rate due to malaria (deaths per 100K people), while grey marks countries with a low mortality rate. (MAP, 2021)

Malaria is endemic in tropical and subtropical areas, mostly in sub-Saharan Africa, but also in the WHO regions of South-East Asia (3% of global malaria deaths), Western Pacific, Eastern Mediterranean, and the Americas (WHO, 2020b). In general, pregnant women and children under the age of five years are the most vulnerable group, but also people with HIV / AIDS (Korenromp *et al.*, 2005) or with immunosuppression as well as travelers have an increased risk to develop severe pathology (Poumerol & Wilder-Smith, 2012).

Malaria is a rural and poverty-related febrile illness (Gallup & Sachs, 2001; Barat *et al.*, 2004). The disease is strongly influenced by climatic conditions, namely temperature, humidity, and rainfall patterns (Gilles, 1993). Climatic factors regulate the development and survival of the mosquito vector and at the same time influence the plasmodial development inside the insect. When temperatures fall below 16°C transmission of the parasite becomes very unlikely since the parasite stops its development and the mosquitoes pause their biting activity (Guerra *et al.*, 2008).

The protozoan parasite is transmitted by females of the *Anopheles* mosquito when taking a blood meal (Ross, 1898). The transmission capacity depends on different factors such as contact frequency between humans and infected insect vector, vector density in general, and feeding habits of the mosquitoes. Besides these parameters, the longevity of the mosquito plays an important role since the transmission frequency is increased when there is more time for the parasite to successfully complete its development inside the mosquito (Breman *et al.*, 2001).

In total, five different *Plasmodium* species are able to infect the human host. The most pathogenic species causing malaria is *P. falciparum* which frequently provokes a severe course of disease and is the reason for most of all malaria deaths. Regional distribution of this parasite species is limited by climatic factors since *P. falciparum* relies on temperatures above 20°C for its development and replication (Anderson & May, 1992; Colluzzi, 1999; Sachs & Malaney, 2002).

Outside the African continent *P. vivax* is the most frequent agent causing malaria. Its wider distribution is based on a lower dependency on temperature when compared to *P. falciparum* (Greenwood *et al.*, 2008; Gething *et al.*, 2012). A limiting issue for *P. vivax* is the restriction to young red blood cells (reticulocytes), leading to a finite maximal parasitemia which reduces the severity of the course of disease (Craik, 1920; HEGNER, 1938; Malleret *et al.*, 2015). *P. vivax* parasites are able to develop into dormant stages in the liver, called hypnozoites, and can persist for months or years in the liver of the patient (White, 2011a; Dembélé *et al.*, 2014). This adaption is ideal in regions with seasonal transmission of malaria.

P. ovale is mainly distributed in tropical Western Africa. In rare instances it is also found in Indonesia, the Philippines, or Papua New Guinea (Kawamoto *et al.*, 1999; Collins & Jeffery, 2005). Similar to *P. vivax*, *P. ovale* infects reticulocytes and has the capacity to develop into dormant hypnozoites that remain in the liver, which can lead to a spontaneous malaria relapse after months.

The fourth *Plasmodium* parasite infecting humans is *P. malariae*. Contrary to *P. vivax* and *P. ovale*, this protozoan species does not generate dormant hypnozoites. Nevertheless, *P. malariae* is capable of remaining in the blood of immune patients over decades without causing symptoms. Co-infections of *P. malariae* and *P. falciparum* are frequent in endemic regions in Africa, but the infection with *P. malariae* often remains undetected until proper laboratory diagnostics are performed (Collins & Jeffery, 2007).

P. knowlesi is the fifth known *Plasmodium* species that can infect humans (Collins, 2012). Originally, it was described in macaques in Malaysia (Franchini, 1927), but experimental work demonstrated that *P. knowlesi* can also infect human beings (Knowles & Gupta, 1932). Recent findings show that the parasite is also naturally transmitted to humans (White, 2008a; Singh & Daneshvar, 2013), where it was often confused with *P. malariae* due to its morphological similarities (Singh *et al.*, 2004).

1.1.3 Pathology

While the development of the parasite inside the liver (see section 1.2.1.2) does not cause any clinical symptoms, the asexual replication of *Plasmodium* species in the erythrocytes is responsible for the symptoms of the disease (Schofield & Grau, 2005; Bartoloni & Zammarchi, 2012). Malaria, particularly if caused by *P. falciparum*, can present in two different ways: it can lead either to uncomplicated malaria or can progress to a severe form. The uncomplicated form of the disease is characterized by non-specific symptoms such as fever, chills, headache, cough, or diarrhea, often resulting in a misdiagnosis. In contrast, severe malaria leads to multiorgan damage, anemia, cerebral malaria, kidney failure, acidosis, or respiratory organ failure (Taylor *et al.*, 2012; Ashley *et al.*, 2018).

The distribution of severe malaria in different age groups in the population is influenced by the transmission rate of the parasite in the different regions. In areas with low transmission of parasite species (low endemicity settings), all ages are at risk of developing clinical symptoms since there is only a low level of immunity in the human population. In these settings, the clinical spectrum of severe malaria is predominated by cerebral malaria. In contrast, when the transmission rate is high (high endemicity settings), the immune system of people adapts to parasite exposure and a status of semi-immunity develops. In these high endemicity regions the constant exposure to parasites prevents severe malaria in adults that become semi-immune due to a history of multiple infections. In contrast, young children often suffer from a severe course of the disease (Cowman *et al.*, 2016).

Besides the transmission rate, severity of a malaria infection is also influenced by the age of the individuals per se, which is due to the maturation status of the immune system of the host. A severe clinical course is mainly present in non-immune children who suffer from symptoms such as severe anemia and hypoglycemia, while adults develop acute pulmonary oedema, kidney failure, jaundice, or cerebral malaria (Dondorp *et al.*, 2008).

The vulnerability for a severe malaria progress is also influenced by the interaction with other infectious diseases. The risk of complicated or severe malaria is intensified when an individual is infected simultaneously with HIV (Korenromp *et al.*, 2005). In contrast, it is assumed that a helminth co-infection shields the individual from severe malaria in some cases, but this seems to be specific for particular geographical sites and modulated by local malaria and helminth infection patterns (Hartgers & Yazdanbakhsh, 2006; Mwangi *et al.*, 2006).

1.2 Biology of *Plasmodium* species

1.2.1 Life cycle of *P. falciparum*

The life cycle of *P. falciparum* parasites includes the shuttling between two different host systems. It consists of the sexual reproduction within the *Anopheles* mosquito vector (section 1.2.1.1), asexual replications in the human host (section 1.2.1.2 and section 1.2.1.3) and the generation of sexual precursors, also in the human host (section 1.2.1.4). The asexual multiplication in the human host involves two separate phases, the liver stage (section 1.2.1.2), and the blood stage (section 1.2.1.3) (Figure 2).
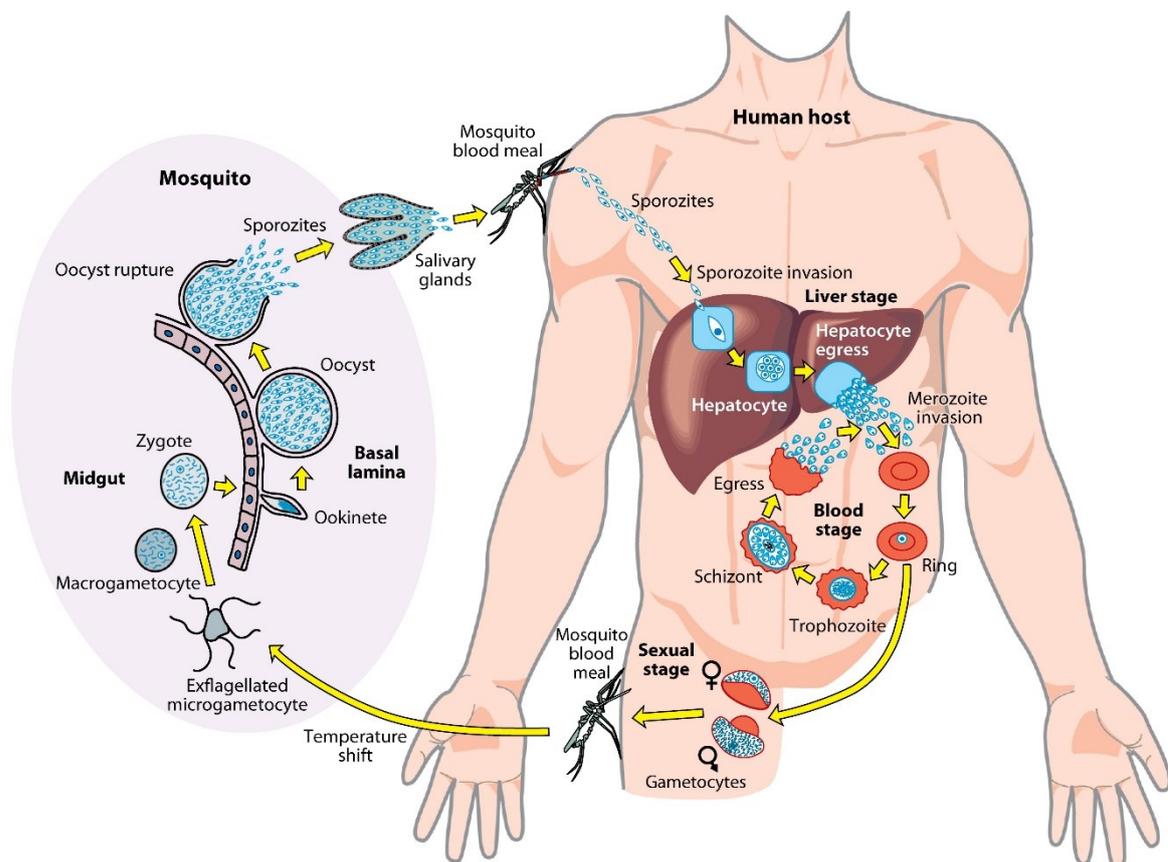
Figure 2) Life cycle of *P. falciparum* parasites. The life cycle of *Plasmodium* parasites includes the shuttling between two hosts, namely the invertebrate (female *Anopheles* mosquito) as well as the vertebrate host (human). During the blood meal of the female *Anopheles* mosquito sporozoites are injected into the human skin and migrate to the liver where the asexual replication inside hepatocytes begins. Mature merozoites are released into the blood stream, infect red blood cells, and start the asexual replication, running through ring, trophozoite and schizont stage. Upon rupture of the host cell up to 32 new daughter merozoites are released, each initiating a new replication round in red blood cells, a process that repeats every 48 hours. Some of the parasites differentiate into the sexual female and male gametocyte stage parasites and can be taken up by another female *Anopheles* mosquito during blood feeding. Inside the mosquito midgut, the parasite undergoes

sexual replication, producing the ookinete which translocates through the midgut wall, arresting in the mosquito midgut basal lamina. During this arrest, the ookinete matures into the oocyts, which results in the formation of sporozoites. The sporozoites are released into the mosquito hemolymph and migrate to the salivary glands where they are stored until the next blood meal of the *Anopheles* mosquito, closing the life cycle of the *Plasmodium* parasite. (Figure from (Boddey & Cowman, 2013))

1.2.1.1 Sexual reproduction in the mosquito vector

The sporozoites, the parasite stage capable to infect the vertebrate host, are produced in a process called sporogony (Sinden, 2015). Sporogony starts, when the female *Anopheles* mosquito ingests the sexual precursor stages (gametocytes) of the *Plasmodium* parasite during a blood meal which then reach the mosquito midgut (Figure 2). In the midgut the male and female gametocytes are released from the infected red blood cell and differentiate into eight exflagellated motile microgametes (in case of the male gametocyte) and one macrogamete (in case of the female gametocyte), a process initiated by changes in temperature, pH and by the presence xanthurenic acid (Billker *et al.*, 1998; Billker *et al.*, 2000; Billker *et al.*, 2004). By fusion of a microgametes and a macrogamete, a zygote is produced, representing the only diploid parasite stage of the *Plasmodium* life cycle (Maccallum, 1897; Sinden *et al.*, 1976; Aikawa *et al.*, 1984). Subsequently, the fertilized zygote develops into a motile ookinete which travels through the epithelium of the midgut wall and differentiates into an oocyst. The oocyst asexually replicates and produces sporozoites. After oocyst rupture the sporozoites exit into the mosquito haemolymph (Sinden, 1974; Aly *et al.*, 2009). Through the haemolymph some sporozoites reach the salivary glands and can be injected into the human host during the next blood meal.

1.2.1.2 Asexual replication in the human liver

The replication within the human host begins when the infectious stages of the sporozoites (section 1.2.1.1) are injected into the human skin during a blood meal of a female *Anopheles* mosquito (Medica & Sinnis, 2005; Prudêncio *et al.*, 2006) (Figure 2). Aided by their gliding motility they reach the human vasculature to enter the blood stream (Stewart & Vanderberg, 1988; Sultan *et al.*, 1997). Transported via the blood circulation, sporozoites reach the liver and - after crossing different types of host cells, including Kupffer cells or sinusoidal endothelial cells - they establish a productive hepatocyte infection (Mota *et al.*, 2001; Pradel & Frevert, 2001; Frevert *et al.*, 2006; Tavares *et al.*, 2013). This process is of migrating through different cell types is called traversal and represents the starting point of the liver

stage. During invasion into the hepatocyte a parasitophorous vacuole (PV) is formed (Meis *et al.*, 1983). Inside this PV the parasite completes many rounds of asexual multiplication, resulting in the production of thousands of merozoites in a process termed schizogony. *P. vivax* or *P. ovale* also produce persistent dormant stages (hypnozoites) during this liver stage. These hypnozoites arrest early in liver development and remain silent up to years after which they activate and complete liver schizogony (Cogswell, 1992). The mature merozoites are packed into vesicles, called merosomes, and bud from the infected hepatocyte to be released into the bloodstream (Sturm, 2006). Upon rupture of the merosomes, the merozoites are released into the blood stream and invade red blood cells, initiating the erythrocyte cycle.

1.2.1.3   Asexual replication in human red blood cells

The invasion of erythrocytes into red blood cells is a process of multiple, highly orchestrated steps (Figure 3) (Gilson & Crabb, 2009; Riglar *et al.*, 2011; Weiss *et al.*, 2015). First, the merozoite attaches to the erythrocyte surface and reorientates its apical pole (harboring the secretory organelles that are needed for invasion (see section 1.2.2.2.1)), towards the red blood cell membrane. For the attachment to the erythrocyte surface different parasite proteins are required, including the erythrocyte-binding ligands (EBLs) and the *P. falciparum* reticulocyte binding protein-binding homologues (*Pf*Rh) (Rayner *et al.*, 2001; Gilberger *et al.*, 2003; Riglar *et al.*, 2011; Srinivasan *et al.*, 2011). During the second step, the parasite forms a tight junction between itself and the host cell. The active invasion process is powered by an actomyosin motor, during which the parasite forces itself into the erythrocyte (Keeley & Soldati, 2004; Baum *et al.*, 2006; Besteiro *et al.*, 2011; Riglar *et al.*, 2011). Simultaneously, the merozoite specific rhoptry organelles release their lipid-rich content to contribute - together with invaginated red blood cell membrane - to the formation of the parasitophorous vacuole membrane (PVM) that surrounds the parasite inside the host cell after invasion is complete (Cowman & Crabb, 2006). After successful invasion, the last step includes the resealing of erythrocyte membrane and red blood cell echinocytosis (Gilson & Crabb, 2009).
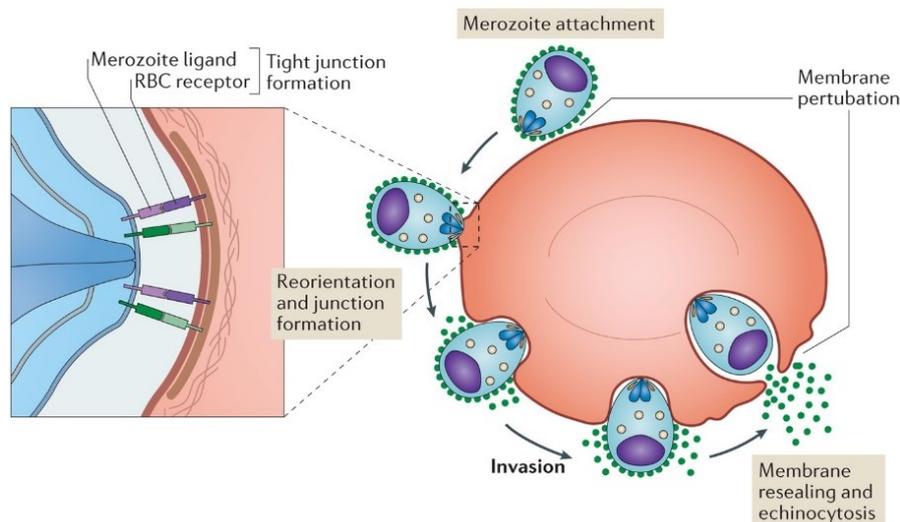
Figure 3) Invasion of the red blood cell. (Right) graphical representation of the different steps of invasion of the merozoite into a red blood cell. (left) enlargement of the apical end of the invading merozoite into the host cell. *Blue*, merozoite; *red*, red blood cell; *green*, merozoite surface proteins. (Niz *et al.*, 2017)

The asexual replication of *P. falciparum* parasite inside the red blood cell lasts 48 hours and during this process, the parasite runs through different stages (Figure 4). The development starts at the ring stage that is important for the parasite to establish itself in the host cell, for instance to install nutrient transport capacities at the host cell membrane. During this stage, the parasite is mobile in the red blood cell and switches between a circular disc shape and an amoeboid form (Grüring *et al.*, 2011). Host cell remodeling is a prominent process during the ring stage and is achieved by the export of many different parasite proteins into the red blood cell (see section 1.2.2.1). The ring stage is followed by the trophozoite stage during which the parasite settles at a stationary position in the host cell and the metabolism increases due to rapid growth of the parasite inside the red blood cell (Figure 4) (Grüring *et al.*, 2011). This results in a higher consumption of nutrients but also increases the need for space in the host cell. Thus, the uptake of host cell cytosol (mostly hemoglobin) is increased. This also leads to an increased formation of haemozoin, a degradation product of hemoglobin that becomes apparent in the parasite's food vacuole (FV) (the site where the hemoglobin is degraded). The trophozoite stage is followed by the schizont stage, where the parasite generates up to 32 new daughter cells (merozoites) within the same cell in a process termed schizogony. Upon rupture of the host cell, these merozoites are released into the blood stream and are able to invade new red blood cells.
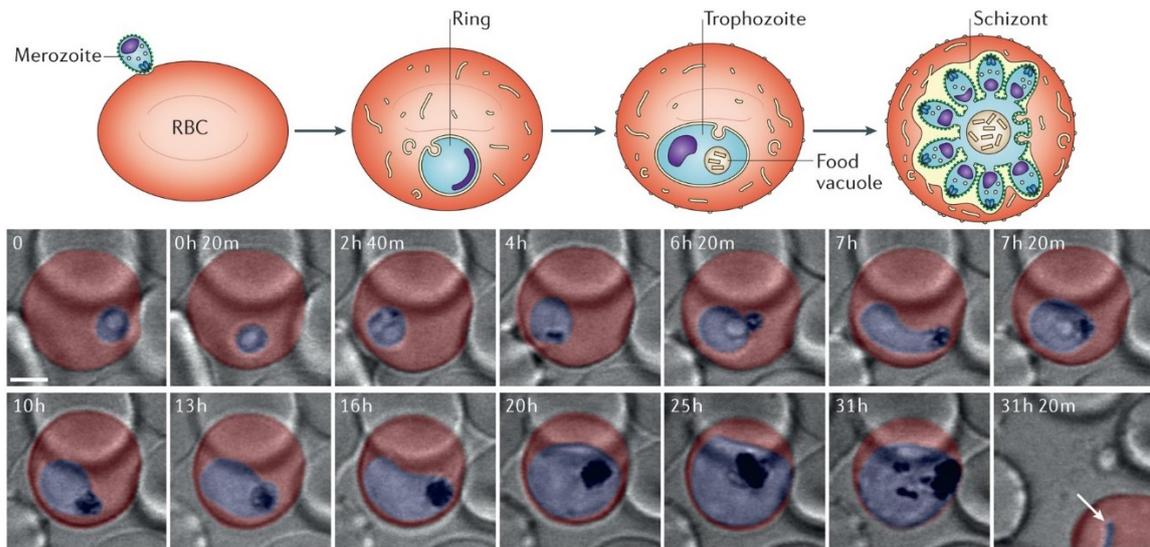
Figure 4) Asexual replication of *P. falciparum* inside the erythrocyte. (Top) Graph displays the different developmental stages of *P. falciparum* inside the host cell, including ring stage, trophozoite stage, schizont stage and merozoites. (Bottom) Confocal microscopy images showing selected single sections of a time lapse experiment of the intraerythrocytic development of a *P. falciparum* parasite. *Red*, marks the red blood cell (top and bottom panel). *Blue*, represents the parasite (top and bottom panel). *Black* shows hemozoin crystals accumulating in the parasite food vacuole (bottom panel). *White arrow* shows a newly invaded ring (bottom panel). (Niz *et al.*, 2017)

1.2.1.4 Development of sexual precursors in the human host

During the repeated rounds of asexual replication inside the red blood cell, some parasites (~ 0.1 – 5%) enter sexual commitment and start the differentiation into gametocytes (Sinden, 1983). This process is called gametocytogenesis and consists of five different morphological stages (Figure 5). The maturation time is species dependent. For *P. falciparum* parasites the differentiation into mature gametocytes requires 9 – 12 days (Hawking *et al.*, 1971; Sinden *et al.*, 1978). It was suggested by different studies that differentiation of immature gametocytes takes place in the bone marrow and spleen, while the mature stages circulate in the blood (Farfour *et al.*, 2012; Aguilar *et al.*, 2014; Joice *et al.*, 2014; Lee *et al.*, 2018; Niz *et al.*, 2018; Obaldia *et al.*, 2018). Gametocytogenesis is regulated by the interplay of different genetic, epigenetic and environmental parameters. On a genetic level ApiAP2-G activates transcription to initiate sexual commitment and this is conserved across *Plasmodium* species (Kafsack *et al.*, 2014; Sinha *et al.*, 2014). Epigenetic silencing of *ap2-g* in asexual blood stage parasites is achieved by interaction of heterochromatin protein 1 (HP1) and the histone deacetylase 2 (HDA2) (Brancucci *et al.*, 2014; Coleman *et al.*, 2014). HP1 can be downregulated through the interaction of the perinuclear protein gametocyte development 1 (GDV1), resulting in the continued

transcription of the *ap2-g* locus (Eksi *et al.*, 2012; Filarsky *et al.*, 2018). The genetic and epigenetic factors are influenced by environmental parameters such as the level of human serum phospholipid lysophosphatidylcholine (LysoPC), all together regulating the differentiation into gametocytes (Brancucci *et al.*, 2017). When gametocytogenesis is completed, the mature gametocytes are present in the human blood stream for several days to increase the chances to be transmitted to an *Anopheles* mosquito (Smalley & Sinden, 1977; Gautret & Motard, 1999; Eichner *et al.*, 2001). When the gametocytes are ingested by the *Anopheles* vector during a blood meal, the *Plasmodium* life cycle is completed.
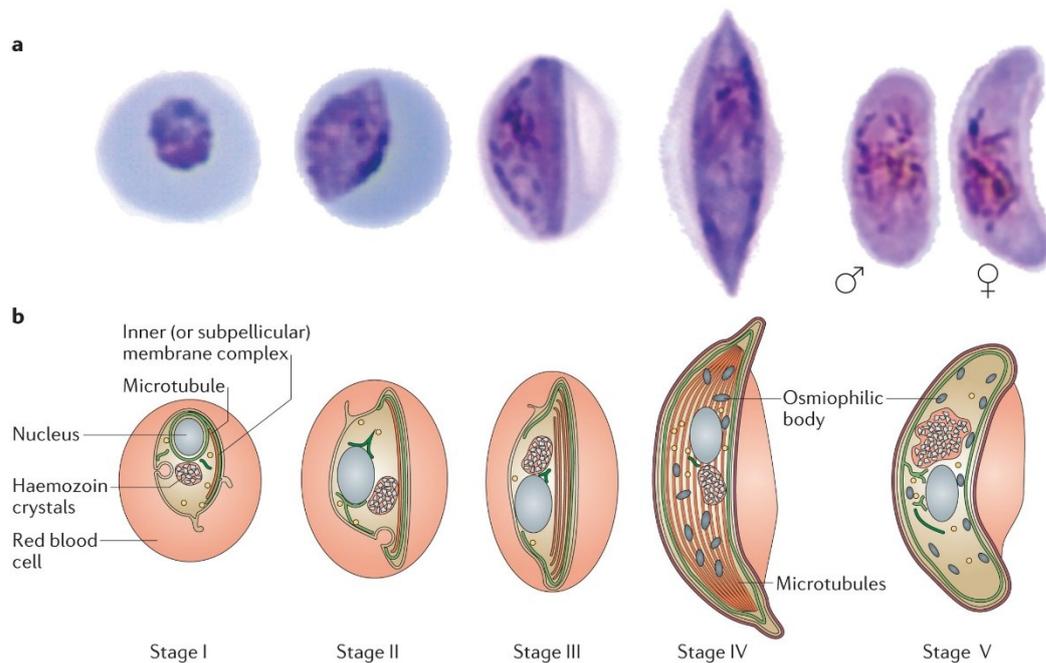
Figure 5) Five stages of gametocytogenesis. (A) The different gametocyte stages from Giemsa-stained smears. (B) Scheme showing the intracellular morphology of the different gametocyte stages during gametocytogenesis. (Josling & Llinás, 2015)

1.2.2 Surviving inside the host cell

1.2.2.1 Remodeling of the infected red blood cell

1.2.2.1.1 The parasitophorous vacuole

The enucleated, metabolic inactive erythrocyte provides the perfect hiding place for the parasite to avoid clearance by the host immune system. Since red blood cells (RBCs) are

not able to present parasite antigens on their surface due to a lack of major histocompatibility complex class I and II, the infected cell remains undetected by the adaptive immune system. Inside the host cell, the parasite establishes the PV and resides inside this special compartment that is surrounded by the PVM, representing the interface between the parasite and the host cell cytoplasm. The PV in *P. falciparum* was described for the first time by *Ladda et al* in 1966 (Ladda *et al.*, 1966). During the entire intraerythrocytic development, the *Plasmodium* parasite replicates inside of the PV, while at the end of the asexual cycle the PVM and the RBC membrane are destroyed by the parasite to release the merozoites into the human blood stream (Blackman & Carruthers, 2013). The PVM serves as a barrier between the erythrocyte and the parasite and regulates the export of proteins and the uptake of nutrients (Garnham *et al.*, 1961; Rudzinska *et al.*, 1965; Aikawa *et al.*, 1966; Aikawa, 1971; Seed *et al.*, 1976). Only little is known about the function of the PV and PVM, but it is suggested that these structures are involved in protein folding and processing since different proteases and chaperons were identified in the PV (Nyalwidhe & Lingelbach, 2006; Spielmann *et al.*, 2012).

1.2.2.1.2 The tubovesicular network

Several studies detected a loop-shaped structure, extending from the PVM and reaching into the erythrocyte cytoplasm, which is termed the tubovesicular network (Kara *et al.*, 1988; Behari & Haldar, 1994; Elford *et al.*, 1995; Elford *et al.*, 1997; Grützke *et al.*, 2014). The role of this structure is not fully defined but it is suggested to be important for nutrient uptake, or possibly is involved in lipid storage necessary for PVM expansion, or senses environmental changes in the erythrocyte cytosol (Lauer *et al.*, 1997; Sherling & van Ooij, 2016).

1.2.2.1.3 The PTEX complex

Enveloped inside the PV, the parasite starts the remodeling of the RBC by exporting many different parasite proteins into the host cell to gain access to nutrients, alter the physical structures of the erythrocyte membrane, and enable cytoadhesion of the infected cell (Sargeant *et al.*, 2006; Maier *et al.*, 2008; Haase & Koning-Ward, 2010). Around 10% of the parasite proteome are exported from the *Plasmodium* parasite to the host RBC (Koning-Ward *et al.*, 2016). Protein export includes three subsequent steps and is mediated either via special export motifs (*Plasmodium* export element, PEXEL or host targeting, HT) that

are processed in the endoplasmic reticulum (ER) or functions without a PEXEL motif (PEXEL-negative exported proteins, PNEPs) (Hiller *et al.*, 2004; Marti *et al.*, 2004; Chang *et al.*, 2008; Osborne *et al.*, 2010; Russo *et al.*, 2010; Spielmann & Gilberger, 2010; Heiber *et al.*, 2013). After entry into the ER, the exported proteins pass through the secretory pathway, after which they cross the parasite plasma membrane (PPM) to reach the PV lumen. To cross the PVM, the exported proteins are translocated into the host cell by a parasite complex, called *Plasmodium* translocon of exported proteins (PTEX) (Koning-Ward *et al.*, 2009; Beck *et al.*, 2014; Elsworth *et al.*, 2014). This PTEX complex consists of five elements of which the AAA+ ATPase heat shock protein 101 (HSP101), the exported protein 2 (EXP2), and PTEX150 represent the core units (Koning-Ward *et al.*, 2009). Beside these three main components, the complex is supported by two accessory parts, thioredoxin 2 (TRX2) and PTEX88 which are dispensable for the parasite during the asexual blood stage replication (Matthews *et al.*, 2013; Matz *et al.*, 2013). After translocation through PTEX, the exported proteins either remain in the erythrocyte cytoplasm or are transported to different destinations in the host cell, such as the erythrocyte plasma membrane or membrane skeleton, or vesicular structures called Maurer´s clefts that are important for protein sorting (Wickham *et al.*, 2001).

1.2.2.1.4 The Maurer`s clefts

Once the exported proteins reach the erythrocyte cytoplasm, the transport to the erythrocyte membrane is aided either by chaperone mediated complexes or the Maurer´s clefts (MC) (Maier *et al.*, 2009). The Maurer's clefts are disk-shaped, single-membrane platforms that are believed to act as a sorting compartment inside the host cell (Lanzer *et al.*, 2006; Bhattacharjee *et al.*, 2008; Mundwiler-Pachlatko & Beck, 2013). The Maurer´s clefts are built 2 – 4 hours after RBC invasion and are mobile in the host cell throughout ring stage development but become stationary during the transition to the trophozoite stage. Immobilization of the Maurer´s clefts is achieved by tether-like structures or by actin filaments (Cyrklaff *et al.*, 2011; McMillan *et al.*, 2013). Starting from the first moment they become detectable, the number of Maurer´s clefts remains constant across the asexual development (Hanssen *et al.*, 2008; Tilley *et al.*, 2008; Grüring *et al.*, 2011; McMillan *et al.*, 2013). Various studies revealed the presence of different integral and peripheral proteins at the Maurer´s clefts, i.e. the membrane associated histidine -rich protein 1 (MAHRP1), the skeleton binding protein 1 (SBP1), the ring-exported protein 2 (REX2), and the merozoite surface related protein 6 (MSRP6) (Blisnick *et al.*, 2000; Spycher *et al.*, 2003; Spielmann *et*

al., 2006; Heiber *et al.*, 2013). Beside these, one of the most prominent Maurer's clefts proteins is the ring exported protein 1 (REX1) which prevents stacking of the Maurer´s clefts and is needed for the transfer of *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), the main parasite virulence protein, to the host cell surface (McHugh *et al.*, 2015). Another important protein is the MC-associated PTP1 (*Pf*EMP1 trafficking protein) that organizes the transfer of *Pf*EMP1 and of STEVOR (subtelomeric variant open reading frame) to the erythrocyte surface (Kaviratne *et al.*, 2002; Przyborski *et al.*, 2005; Cyrklaff *et al.*, 2011).

1.2.2.1.5 Knob structures at the erythrocyte surface are needed for parasite virulence

The knobs are surface elevations generated during trophozoite development via the accumulation of knobs-associated histidine-rich protein (KAHRP) and are connected to the erythrocyte cytoskeleton (Rug *et al.*, 2006; Watermeyer *et al.*, 2016). The surface antigens *Pf*EMP1 and STEVOR are displayed at these knob structures present at the erythrocyte membrane (Kriek *et al.*, 2003; Tilley *et al.*, 2008). *Pf*EMP1 mediates the cytoadhesion of the infected red blood cell to the endothelium, thus promoting sequestration to the human endothelium to avoid the elimination by the spleen (Kilejian, 1979; Baruch *et al.*, 1995). Most severe complications appear during *P. falciparum* infections due to this sequestration ability (Beeson & Brown, 2002; Miller *et al.*, 2002; Wassmer *et al.*, 2015; Plewes *et al.*, 2019). Besides this, due to the protein-export driven reorganization of the cytoskeleton and the generation of the cytoskeleton-anchored knob structures, the erythrocyte membrane becomes stabilized and inflexible, further enhancing trapping in small capillaries (Zhang *et al.*, 2015).

1.2.2.2 *Plasmodium* specific organelles

Plasmodium parasites are unicellular eukaryotes and possess different organelles such as the endoplasmic reticulum (ER), a rudimentary Golgi apparatus, ribosomes, the apical organelles, and two organelles of endosymbiontic origin, the mitochondrion and a non-photosynthetic plastid called apicoplast (McFadden *et al.*, 1996; van Wye *et al.*, 1996; Bannister *et al.*, 2000; Bannister *et al.*, 2004; Struck *et al.*, 2005) (Figure 6).

A

B
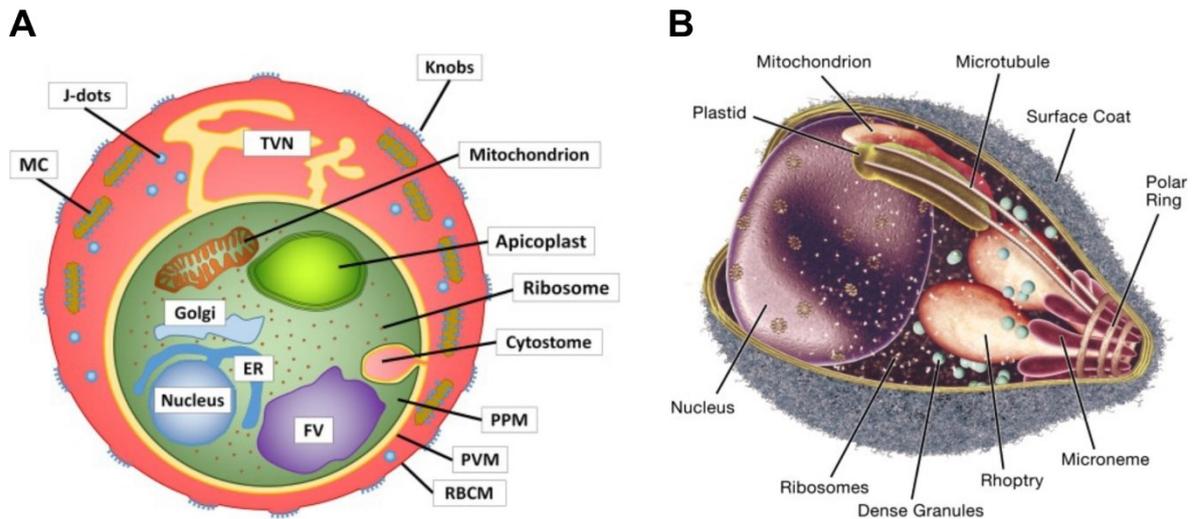
Figure 6) Intracellular organelles of trophozoite stage and merozoite stage *Plasmodium* parasite. (A) Scheme depicting the intracellular morphology of a trophozoite stage parasite. Different organelles are shown. MC, Maurer´s clefts; TVN, tubovesicular network; ER, endoplasmic reticulum; FV, food vacuole; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; RBCM, red blood cell membrane. (B) Graph showing the different organelles of the invasive merozoite stage parasite. (modified from (Cowman & Crabb, 2006; Flammersfeld *et al.*, 2018))

1.2.2.2.1 The apical organelles of the invasive stages

The life cycle of *Plasmodium* parasites is characterized by different invasive stages capable to invade the host cells, it develops in during different life cycle phases. To enable the invasion of the host cell, the parasites possess special essential apical organelles that are situated at the anterior end of the invasive stages. These include the rhoptries, micronemes and dense granules (Sam-Yellowe, 1996; Bannister *et al.*, 2000; Cowman & Crabb, 2002). The function and development of the apical organelles was initially investigated and characterized in *P. knowlesi,* mainly by electron microscopy (Bannister *et al.*, 1975; Aikawa *et al.*, 1978; Bannister *et al.*, 1986; Bannister & Mitchell, 1989; Torii *et al.*, 1989). The rhoptries are club-shaped elongated organelles (Bannister *et al.*, 2000). These organelles are present in pairs in the invasive stages and are filled with granular material. The secretion of their content, e.g. different rhoptry neck proteins (RONs) is essential for the invasion into the host cell (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). In addition to proteins needed for invasion, the rhoptries also secrete proteins that are incorporated into the PVM during its formation (Sam-Yellowe *et al.*, 1988; Beckers *et al.*, 1994). The micronemes are also found at the apical end of the invasive stages. Like the rhoptries, they contain different types of

proteins involved in invasion, e.g. the apical membrane antigen 1 (AMA-1). For instance, the molecular interplay between RON2 and AMA-1 was shown to be a critical step of the invasion process (Lamarque *et al.*, 2011). The third component of the apical complex are the dense granules which are electron dense matrix granules (Aikawa *et al.*, 1990; Culvenor *et al.*, 1991; Trager *et al.*, 1992; Bannister *et al.*, 2000). In *P. falciparum* examples of dense granule proteins are the ring-infected erythrocyte surface antigen (RESA), ring membrane antigen (RIMA), and subtilisin-like serine proteases (SUBs) (Aikawa *et al.*, 1990; Culvenor *et al.*, 1991; Trager *et al.*, 1992; Blackman *et al.*, 1998). When the parasite invades the host erythrocyte, the content of the dense granules is released into the PV lumen (Preiser *et al.*, 2000; Blackman & Bannister, 2001).

1.2.2.2.2 The apicoplast

Another *Plasmodium* specific organelle is the four membrane-bounded apicoplast that is found in most Apicomplexan parasites, such as *Plasmodium* and *Toxoplasma* but is lacking e.g. in *Cryptosporidium* (Zhu *et al.*, 2000). The apicoplast was acquired by endosymbiosis of a red algae (McFadden *et al.*, 1996). This organelle is of cylindrical shape that develops into a highly branched compartment in later *Plasmodium* parasite stages (Hopkins *et al.*, 1999; Waller *et al.*, 2000; Waller & McFadden, 2005). The apicoplast hosts different metabolic pathways, namely the biosynthesis of heme, type II fatty acids and isoprenoid precursors (Ralph *et al.*, 2004; Yu *et al.*, 2008; Vaughan *et al.*, 2009). However, during asexual replication in the erythrocyte only the isoprenoid precursor pathway is essential for parasite development (Yeh & DeRisi, 2011). Based on the fact that neither humans, nor animals possess a plastid, the apicoplast represents a very interesting target for drug development against apicomplexan causing diseases in humans and livestock. Electron and fluorescence microscopy analyses suggest a close association between the apicoplast and the mitochondrion in different erythrocytic stages (Aikawa, 1966; Hopkins *et al.*, 1999; Ralph *et al.*, 2004; Tonkin *et al.*, 2004). Both organelles, mitochondrion and apicoplast are not synthesized *de novo* during parasite replication but have to be replicated during growth and distributed between the daughter cells.

1.2.2.2.3 The food vacuole

The food vacuole (FV) or digestive vacuole (DV) is an acidic, lysosome-like organelle (Gluzman *et al.*, 1994; Francis *et al.*, 1997; Lazarus *et al.*, 2008; Abu Bakar *et al.*, 2010). The FV likely is present during the entire cycle of the parasite but is most easily detected by the accumulating hemozoin which becomes prominent in early trophozoite stage parasites in the blood phase (Abu Bakar *et al.*, 2010; Grüring *et al.*, 2011). The main function of the FV is the degradation of hemoglobin which also requires heme and oxygen radical detoxification, and the FV also has functions in the storage of non-degradable biomolecules, the modulation of intracellular ion homeostasis of the host erythrocyte, and peptide and amino acid transport (Goldberg *et al.*, 1990; Olliaro & Goldberg, 1995; Staines *et al.*, 2001; Krugliak *et al.*, 2002; Lew *et al.*, 2004). Since it plays a central role in the metabolism of the parasite, it serves as a main target for many antimalarial drugs. Host cell cytosol is taken up via the cytostome, a structure that is formed through the invagination of the PPM and the PVM (Aikawa *et al.*, 1966; Langreth *et al.*, 1978; Slomianny, 1990; Goldberg, 1993). When the parasite takes up hemoglobin, double-membranous vesicles arise from the cytostome and are transferred to the FV, where the hemoglobin is degraded (Slomianny, 1990; Goldberg, 1993; Spielmann *et al.*, 2020). Hemoglobin degradation is very important for the parasite since this process provides amino acids for the *de novo* synthesis of proteins and amino acids are also important metabolites for many pathways (Goldberg, 2013; Krishnan & Soldati-Favre, 2021). During the proteolytic degradation of hemoglobin, toxic, heme byproducts are produced that must be neutralized by the parasite via oligomerization into the chemical unreactive hemozoin crystals (Pagola *et al.*, 2000; Banerjee, 2001; Egan *et al.*, 2002; Goldberg, 2005; Egan, 2008). To proteolytically process hemoglobin, several enzymes are necessary. The first steps are performed by aspartic proteases called Plasmepsins and the cysteine proteases falcipains, followed by the metalloprotease falcilysin and the dipeptidyl aminopeptidase 1 (*Pf*DPAP1) (Goldberg *et al.*, 1990; Eggleson *et al.*, 1999; Klemba *et al.*, 2004). As not all amino acids can be produced by the parasite, the remaining amino acids that are either present in a limited amount (methionine, cysteine, glutamic acid and glutamine) or even absent (isoleucine) in hemoglobin must be taken up by the parasite from extracellular sources to fully cover the supply with amino acids (Divo *et al.*, 1985; Goldberg, 2005; Liu *et al.*, 2006). Besides the metabolic role of hemoglobin uptake and digestion, the parasite also uses endocytosis to gain space inside the host cell and prevent RBC lysis by regulating red blood cell homeostasis (Ginsburg, 1990; Lew *et al.*, 2003). To transfer the different nutrients across the FV membrane and to maintain the acidic environment inside the FV, different transporter proteins are present in the FV

membrane. This includes the multi-drug resistance protein 1 (*Pf*MDR1) and the chloroquine resistance transporter (*Pf*CRT), which are important for the import of soluble molecules, or the export of metabolites (Martin & Kirk, 2004; Rohrbach *et al.*, 2006; Shafik *et al.*, 2020). In both cases, variations of the transporters are associated with drug resistance (Fidock *et al.*, 2000; Reed *et al.*, 2000; Picot *et al.*, 2009). The ion homeostasis in the FV and inside the parasite is balanced by the V-type H+-ATPase and the V-type H+-PPase (*Pf*VP1) (McIntosh, 2001; Saliba *et al.*, 2003).

1.3 Malaria Prophylaxis and Treatment

1.3.1 Vector control and drug-based prevention of malaria

To combat malaria transmission and disease, vector control as well as chemical prevention measures are important tools of intervention. The currently most frequently used vector control measures has the aim to impede human – mosquito contact and comprise insecticide-treated nets (ITNs), untreated nets (UN), indoor residual spraying (IRS) (WHO, 2017c; Wangdi *et al.*, 2018). There are two different types of ITNs on the market: for the long-lasting insecticidal nets (LLINs) the insecticide is integrated in the fibers during the production process, which reduces the washing-off of the insecticide and prolongs protection. The second type of mosquito nets are the insecticide-treated nets, which need to be repeatedly impregnated with insecticide every six months to maintain their preventive character. For net impregnation pyrethroids are used, but unfortunately resistance against this type of insecticide is raising worldwide, including on the African continent (Chandre *et al.*, 1999; Hargreaves *et al.*, 2000; Hargreaves *et al.*, 2003; Stump *et al.*, 2004; Etang *et al.*, 2006; Glunt *et al.*, 2015). Nevertheless, pyrethroid impregnated ITNs currently still offer efficient protection against the mosquito vector (Curtis *et al.*, 2003; Curtis *et al.*, 2006; Wamae *et al.*, 2015; Wangdi *et al.*, 2016).

Beside the ITNs, IRS is also a highly effective method to prevent malaria transmission. For IRS, the house walls are sprayed periodically with insecticides to kill the *Anopheles* mosquito resting on the walls and this can also prevent the mosquitoes from entering the house (WHO, 2006; Hamusse *et al.*, 2012).

Another method to prevent malaria is the administration of chemoprophylactic drugs. Chemoprophylactic drugs are mainly given to non-immune, healthy persons, traveling from nonendemic to malaria endemic regions. The main administered prophylactic drugs used

today are sulphadoxine-pyrimethamine (SP), mefloquine (MQ), amodiaquine (AQ), dihydroartemisinin-piperaquine (DP) and artesunate (AS) (White, 2005; Greenwood, 2010). SP is the most widely administered drug, which resulted in an increase in resistance to this drug and treatment failures (Rønn *et al.*, 1996; Roper *et al.*, 2004; Mugittu *et al.*, 2005; Gosling *et al.*, 2009; Tan *et al.*, 2014). To counteract this trend, mefloquine and amodiaquine are more intensively used, either as a substitutional treatment or in combination with SP (Clerk *et al.*, 2008; Gosling *et al.*, 2009).

Overall, ITNs are still the most effective measure to reduce malaria transmission, since chemoprophylactic drug administration harbors certain uncertainties regarding i.e., administration policy, limitations in preventing *P. vivax* relapses, or severe side effects. Nevertheless, a combination of all prophylactic measures provides the highest protection and contribute to malaria elimination (Wangdi *et al.*, 2018).

1.3.2 Vaccines

Eliminating malaria is a challenging task that will likely only be achieved with the aid of a potent vaccine. Ideally, a vaccine would initiate an immune response against all life cycle stages of *Plasmodium*, inhibiting both transmission and clinical disease. In collaboration with the world´s leading funders of malaria vaccine the WHO set up the Malaria Vaccine Technology Roadmap which is constantly evaluated and revised (PATH's Malaria Vaccine Initiative, 2015). The latest version of this roadmap has the aim to implement a global strategy to accelerate the development of an efficient malaria vaccine against *P. falciparum* and / or *P. vivax*. The overall goal is to design a next-generation vaccine with a protective efficiency of at least 75% and an immune response lasting for up to 2 years (Moorthy *et al.*, 2013).

1.3.2.1 Pre-erythrocytic vaccines

The immune response triggered by a potential vaccine can target different developmental stages of the *Plasmodium* life cycle (Figure 7). Antigens of the invasive sporozoites or of liver stage parasites are targeted by the pre-erythrocytic vaccines (PEV), which are also called anti-infection vaccines. These vaccines activate the adaptive immune system via different surface antigens to produce antibodies or T cell responses to combat sporozoites present in the blood stream and skin or deplete infected hepatocytes. Pre-erythrocytic

vaccines can be subdivided into subunit vaccines or whole cell vaccines. Recent research mainly focused on subunit vaccine development based on the major sporozoite surface antigen termed circumsporozoite protein (CSP) (Dame *et al.*, 1984). The RTS,S/AS01E (Mosquirix^{TM}) vaccine incorporating a *P. falciparum* CSP fragment (Asn-Ala-Asn-Pro [NANP]) vaccine belongs to this group and represents the most developed malaria vaccine to date (Cohen, 1996; White *et al.*, 2015). The European Medicine Agency (EMA) approved RTS,S/AS01E to be the first malaria vaccine used in humans, which is currently tested in phase IV (WHO, 2017a). Earlier trials showed a protective efficacy of e.g. ~36% in young children treated with four vaccine doses in sub-Saharan Africa, but also decreasing efficacy was reported in the follow-up period, especially in the absence of a booster dose of the vaccine (RTS, 2014, 2015). A second promising vaccine candidate - R21/MM - was currently developed by the University of Oxford. This candidate is a derivative of RTS,S and is also based on the central NANP repeat of CSP coupled to a saponin-based adjuvant termed Matrix-M (R21/MM) (Datoo *et al.*, 2021). In a randomized controlled trial conducted in Burkina Faso, children aged 5 – 17 months were treated with three doses R21/MM in a 4-week interval before the malaria season followed by a fourth vaccination one year later. Overall efficacy one year later was at 77% and participants had high antibody titers against the central NANP repeat of *Plasmodium*. Based on these findings this might represent a break-through in vaccine development to combat malaria.

Vaccines based on the full-length antigen of pre-erythrocytic stage parasites are the whole sporozoites vaccines (WSV) (Figure 7). Already in the 1970s attention was drawn to this type of vaccine since radiation-attenuated WSV to immunize humans resulted in a high protective efficacy. In these experiments, immune response against the invasive sporozoite stages, but not infection with blood-stage parasites, was demonstrated (Clyde *et al.*, 1973; Clyde, 1975). Three different approaches exist to attenuate sporozoites for WSV: The sporozoites can either be attenuated by radiation (PfSPZ Vaccine), or chemically attenuated by simultaneous administration of an antimalarial drug such as chloroquine (PfSPZ-CVac), or the parasites are genetically engineered to delete genes essential for liver stage development (Richie *et al.*, 2015; Duffy & Patrick Gorres, 2020; Metzger *et al.*, 2020).

1.3.2.2 Blood-stage vaccines

The second type of malaria vaccine, which is called blood-stage vaccines (BSV) (Figure 7), is designed to interrupt the asexual blood-stage replication, and ideally clear the infection. Immunization experiments performed in monkeys in the 1970s directed the interest of the

developers on the merozoite invasion process as a target for vaccine development (Siddiqui, 1977). The design of a merozoite-oriented vaccine faces a number of obstacles. Firstly, invasion into a new red blood cell lasts only a few seconds, such a vaccine therefore has only a very small part of the live cycle as the target. Secondly, merozoites display many surface antigens that are often variable between isolates (polymorphic) and the invasion process depends on different, redundant ligands, complicating the design of an effective vaccine. Thirdly, compared to other vaccine types, merozoite-based BSV need to target a large number of parasites at the same time.

From 2000 to 2015 antigens of MSP1 and AMA1 were the most prominent BSV vaccine targets, but also EBA-175 and MSP3 were tested as antigen candidates. Most of the completed trials registered at ClinicalTrials.gov showed now or only moderate efficiency (Duffy & Patrick Gorres, 2020). In an attempt to obtain merozoite vaccines with better protection levels, new antigen targets such as the lowly polymorphic *P. falciparum* reticulocyte binding protein homologue 5 (*Pf*RH5) or the AMA1-RON2 complex are being developed (Srinivasan *et al.*, 2017).

1.3.2.3 Transmission-blocking vaccines

The third group of antimalarial vaccines are the transmission-blocking vaccines (TBV) (Figure 7). This type of vaccine is used to establish antibodies in the human host that target surface antigens of the sexual mosquito stages (gametes or zygotes). These antibodies are absorbed by the female *Anopheles* mosquito during the blood meal and aim to kill the parasites or prevent its development inside the mosquito to inhibit further transmission to a new host (Carter & Chen, 1976; Gwadz, 1976) . The most promising targets for this type of vaccine are the *P. falciparum* gamete surface proteins Pfs230 and *Pf*s48/45, or the zygote surface proteins *Pf*s25 and *Pf*s28 (Carter & Kaushal, 1984; Grotendorst *et al.*, 1984; Duffy *et al.*, 1993). For TBVs to be efficient in blocking transmission, one obligatory demand is the generation of high and durable antibody titers in humans. This is mostly achievable via specific adjuvants. Another critical requirement for TBVs is the need for a remarkably high safety profile, as the vaccinated human does not benefit directly from the vaccine.
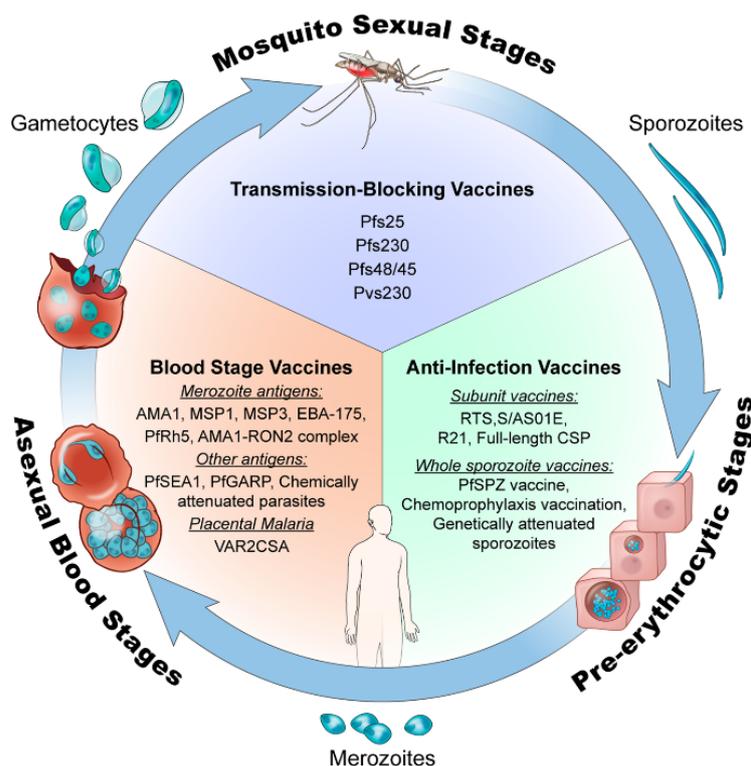
Figure 7) Vaccine targets of *Plasmodium* stages. Scheme describes different developmental stage of *Plasmodium* that can be targeted by vaccine candidates. (Duffy & Patrick Gorres, 2020)

1.3.3   Antimalarial drugs

Antimalarial drugs are a very important tool in the prevention (see section 1.3.1) and therapy of malaria disease. The WHO lists 14 drugs for malaria treatment and six agents for chemoprophylaxis, either applied as single or combination therapy in the WHO Model List of Essential Medicines (WHO, 2019b). Unfortunately, the success of antimalarial drugs is hampered by resistance of the parasite against all of the commercially used drugs. Development of new antimalarials, ranging from the modification of already existing drugs to the *de novo* synthesis of an efficient agent, is very laborious since they must be safe, highly effective, should be easy to apply and must be cheap (Burrows *et al.*, 2017). All these requirements are additionally complicated by the fact that the development of an antimalarial drug is relatively unprofitable for the pharmaceutical industry and hence there is very limited investment into the development of novel antimalarials. In 1999, the "Medicines for Malaria Venture" was founded in Switzerland, a non-profit product development partnership, which has the goal to reduce disease burden in malaria endemic countries (MMV Medicines for Malaria Venture, 2021). In collaboration with different

universities and pharmaceutical companies, this organization develops new antimalarial drugs for the treatment of malaria.

1.3.3.1   Quinoline derivatives

Most of the commonly used drugs target the erythrocytic stages of *Plasmodium* parasites. Currently, there are four major drug classes on the market that target different specific processes of the parasite. The quinoline derivatives comprise a large group of agents (e.g. chloroquine, amodiaquine, quinine, mefloquine, piperaquine, lumefantrine) that are either provided as single treatment or as combination therapy with other antimalarials, e.g. artemisinin derivatives. Quinolines and its derivatives target the hemoglobin digestion in the FV of the *Plasmodium* parasite by preventing the degradation byproduct heme from polymerizing into unreactive hemozoin crystals (Sullivan, 2002). This leads to the accumulation of toxic heme in the FV, causing parasite death. One of the most prominent representatives of this group is quinine, which was isolated from the cinchona tree in 1820 and was the first chemically purified agent used for the therapy of malaria. Another famous member is chloroquine, developed in 1934, which was widely used to treat all forms of malaria and was very well tolerated (F. Loeb *et al.*, 1946). Resistance to this drug was for the first time reported in the 1950s and was shown to be based on mutations in the *P. falciparum* chloroquine resistance transporter (*Pf*CRT), resulting in an increased transport of chloroquine out of the FV (Payne, 1987; Fidock *et al.*, 2000; Wellems & Plowe, 2001).

1.3.3.2   Antifolates

The second class of antimalarials are the antifolates. This group comprises the drugs sulfadoxine-pyrimethamine and atovaquone-proguanil, which target the essential folate metabolism of the parasite (Lumb *et al.*, 2011). Sulfadoxine-pyrimethamine is very inexpensive and thus substituted chloroquine as first-line treatment in various African countries. Unfortunately, widespread resistance results in total failure of the combination therapy (Curtis *et al.*, 1998; Christopher V. Plowe, 2001; Sibley *et al.*, 2001). However, in malaria-endemic African areas sulfadoxine-pyrimethamine is administered as intermittent preventive treatment during pregnancy (White, 2005; Zhao *et al.*, 2020).

1.3.3.3 Atovaquone

Atovaquone is another antimalarial that can be used in combination with the antifolate proguanil (Malarone[TM]) either for prophylaxis or for treatment of malaria (Radloff *et al.*, 1996; Høgh *et al.*, 2000). This compound targets the cytochrome *c* oxidoreductase in mitochondria and results in the disruption of the mitochondrial membrane potential by inhibiting electron transport (Fry & Pudney, 1992; Vaidya & Mather, 2000).

1.3.3.4 Artemisinin and its derivatives

The most potent antimalarial drug at present is artemisinin and its derivatives (ART). Artemisinin is isolated from the plant *Artemisia annua*, a plant that has been used for long time in Chinese traditional medicine (Qinghaosu Antimalaria Coordinating Research Group, 1979). The Chinese researcher Youyou Tu was the first to extract artemisinin as the active ingredient from this herb in the 1970s and demonstrated its use as highly efficient antimalarial (Tu *et al.*, 1981; Tu, 2011). For her extraordinary work she was awarded with the Nobel Prize in Physiology and Medicine in 2015 (NobelPrize.org, 2015). To prevent resistance, artemisinin and its derivatives are provided in a combination therapy with a partner drug with a different mode of action compared to ART. The artemisinin combination therapy (ACT) consists of the short-acting artemisinin derivative (*in vivo* half-life ~ 1 hour in humans), resulting in a first major reduction of the parasite mass and a longer acting partner drug, clearing the remaining parasites circulating in the bloodstream (White, 2008b; Dondorp *et al.*, 2009; Enserink, 2010; White, 2013). The most common used combinations are artemether-lumefantrine, artesunate-amodiaquine and dihydroartemisinin-piperaquine. ACTs are recommended by the WHO as first-line treatment for uncomplicated *P. falciparum* malaria, but they are also used for the treatment of chloroquine-resistant *P. vivax* malaria (WHO, 2015). ACTs show a high efficacy in clearing the asexual blood stage parasites, but they are also active against gametocytes.

The most prominent artemisinin derivatives are artemether, artesunate and arteether, which are semi-synthetic plant prodrugs that are metabolized into the active metabolite dihydroartemisinin (DHA) (Eastman & Fidock, 2009). The antimalarial activity of artemisinin and its derivatives is dependent on an intramolecular endoperoxide bridge. Cleavage of this endoperoxide bridge is induced by hemoglobin degradation products (heme) in the FV which produces free radicals and reactive oxygen species that damage essential parasite proteins and lipids (Meshnick, 2002; Li & Zhou, 2010; Meunier & Robert, 2010; O'Neill *et*

al., 2010; Klonis *et al.*, 2013; Wang *et al.*, 2015; Ismail *et al.*, 2016). This ART activation mechanism is further underlined by different studies, where hemoglobin degradation proteases (falcipains) were inhibited or inactivated, thus leading to a reduced hemoglobin digestion in the FV and subsequently less activated ART (Klonis *et al.*, 2011; Xie *et al.*, 2016). However, resistance to this drug also occurred (section 1.4) and the molecular basis has been elucidated (section 1.4.2).

1.4 Artemisinin resistance

1.4.1   Definition of ART resistance and current situation worldwide

Per definition by the WHO, parasites are resistant to ART when they are still detected by light microscopy on day three after combination therapy treatment or present an increased clearance half-life more than five hours after ACT treatment in the clinic (Flegg *et al.*, 2011; White, 2011b; World Health Organization, 2017). ART resistance is based on a reduced susceptibility of young ring stages to the drug, indicating that it is only a partial resistance (Witkowski & Khim *et al.*, 2013; Dogovski *et al.*, 2015).
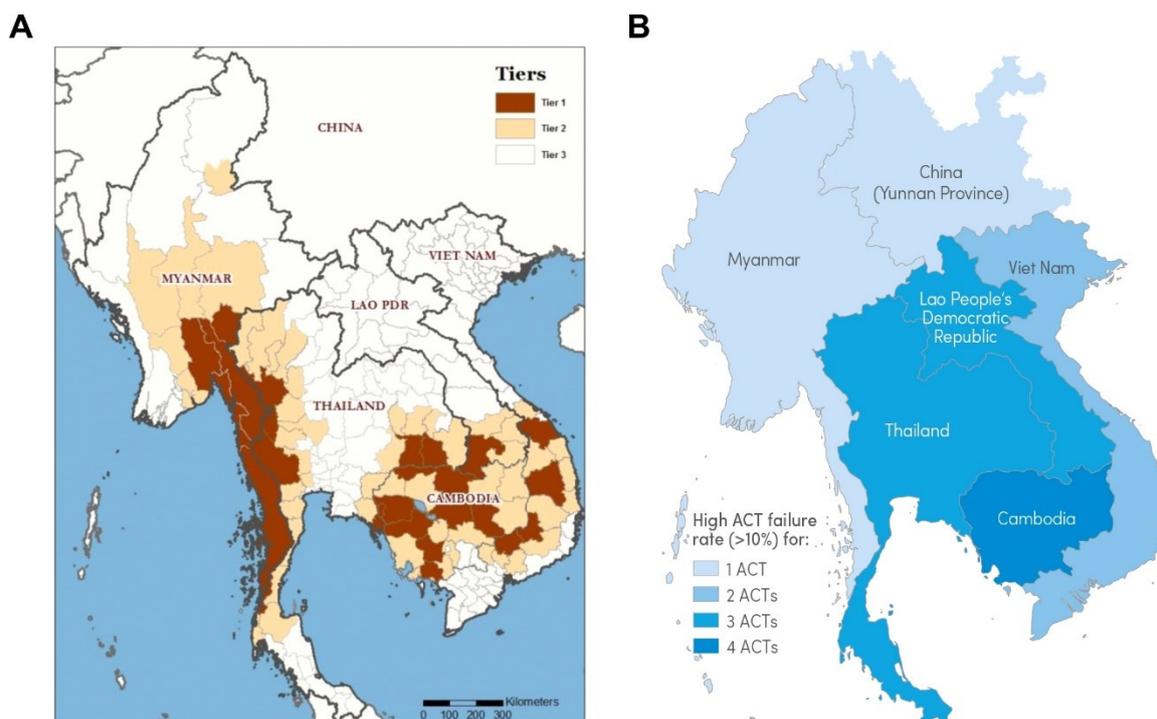
Figure 8) Status of artemisinin resistance in 2014 and 2018. (A) Status of ART resistance in 2014 where resistance was confirmed in five different countries of the Greater Mekong Subregion (GMS). (B) Number of ACTs failing to efficiently treat *P. falciparum* infection in the GMS in 2018 (failure rate > 10%). Currently five different ACTs are recommended by the WHO, and a sixth ACT is positively evaluated by the EMA in regions where all other combination therapies failed. Color code describes the number of failing ACTs in a distinct region. (modified from (WHO, 2014, 2018))

First evidence for ART resistance was found in Pailin, western Cambodia, in 2008 (Noedl *et al.*, 2008). Starting in western Cambodia, the resistance spread over the entire Greater Mekong Subregion (GMS) and by 2014 was also endemic in the Lao People´s Democratic Republic, Myanmar, Thailand, and Vietnam (Dondorp *et al.*, 2009; Amaratunga *et al.*, 2012; Hien *et al.*, 2012; Phyo *et al.*, 2012; Kyaw *et al.*, 2013; Ashley *et al.*, 2014) (Figure 8). Also at that time, a molecular marker of ART resistance was elucidated and confirmed to be causal for resistance in field isolates (see section 1.4.2) (Ariey *et al.*, 2014; Straimer *et al.*, 2015). To determine whether the decreased ART sensitivity developed independently at the different sites or originated from the same parasite strain in western Cambodia, the population structure was assessed based on genome sequencing and transcriptome data of different parasite isolates. These studies indicated that resistance in the GMS arose by both, clonal expansion, and *de novo* from a few founder populations (Miotto *et al.*, 2013; Miotto *et al.*, 2015; Imwong *et al.*, 2017; Zhu *et al.*, 2018). Besides the Southeast Asian subcontinent, ART resistance conferring mutations were also detected with low prevalence in South America and Oceania but at these sites there is little evidence for reduced parasite clearance after ACT treatment and treatment failures (Chenet *et al.*, 2016; WHO, 2019a).

Of major concern was the development of ART resistance in Africa and that resistance might spread from the Southeast Asian continent. For a long time, this seemed not to be the case and ART resistance conferring mutations were only sporadically detected in Africa and were without evidence for reduced parasite clearance or confirmed treatment failure (Mutabingwa *et al.*, 2005; Yeka *et al.*, 2005; Kamya *et al.*, 2007; Yeka *et al.*, 2008; WorldWide Antimalarial Resistance Network (WWARN) Lumefantrine PK/PD Study Group, 2015; Yeka *et al.*, 2016; Sagara *et al.*, 2018; Yeka *et al.*, 2019). One reasons for this may be that the partner drugs are still highly effective in African countries. Other reasons may be the overall high level of the population´s immunity and the multiplicity of infection in malaria endemic African countries (Rosenthal, 2013). As it was shown that ART resistant strains display a reduced fitness level compared to sensitive parasites, resistance would mean a competitive disadvantage for parasites in the polyclonal African host environment (Rosenthal, 2013; Nair *et al.*, 2018). Unfortunately, current studies demonstrated the presence of ART resistance conferring mutations in Rwanda and found evidence for a link of this low prevalent genetic variation with reduced parasite clearance after ACT treatment in a therapeutic efficacy study (Uwimana *et al.*, 2020; Uwimana *et al.*, 2021). However, it was shown that this partial resistance established itself independently and did not spread from the Southeast Asian continent (Chenet *et al.*, 2016; Uwimana *et al.*, 2020).

1.4.2 Molecular markers of ART resistance

1.4.2.1 *Pf*Kelch13 as molecular marker of ART resistance

In 2014, a study performed by *Ariey et al.* uncovered a molecular determinant of ART resistance. These authors selected an ART resistant parasite line by high doses of ART *in vitro* over several years and showed that the non-synonymous single nucleotide polymorphism (SNP) C580Y located in the propeller domain of the *P. falciparum* Kelch13 protein (*Pf*Kelch13; PF3D7_1343700) is associated with delayed parasite clearance after ACT treatment as determined by a decreased ART sensitivity in the ring stage survival assay (RSA) (Ariey *et al.*, 2014). Additionally, mutagenesis studies performed in ART-resistant field isolates underlined the fundamental role of *Pf*Kelch13 in the ART resistance pathway (Straimer *et al.*, 2015). The genomic information encoding the protein *Pf*Kelch13 is located on chromosome 13. The corresponding protein comprises 3 major functional domains (Figure 9): A N-terminal *Plasmodium*-specific sequence, followed by a BTB/POZ domain, a domain that in proteins of a similar domain structure was shown to be a regulator

of ubiquitin-directed degradation, and the C-terminal six blade Kelch propeller repeat that is predicted to serve as platform for protein-protein interactions (Ariey *et al.*, 2014). The C-terminal Kelch propeller region plays a key role in ART resistance. All so far known resistance conferring mutations are located in this repeat domain (WWARN K13 Genotype-Phenotype Study Group, 2019) (Figure 9).
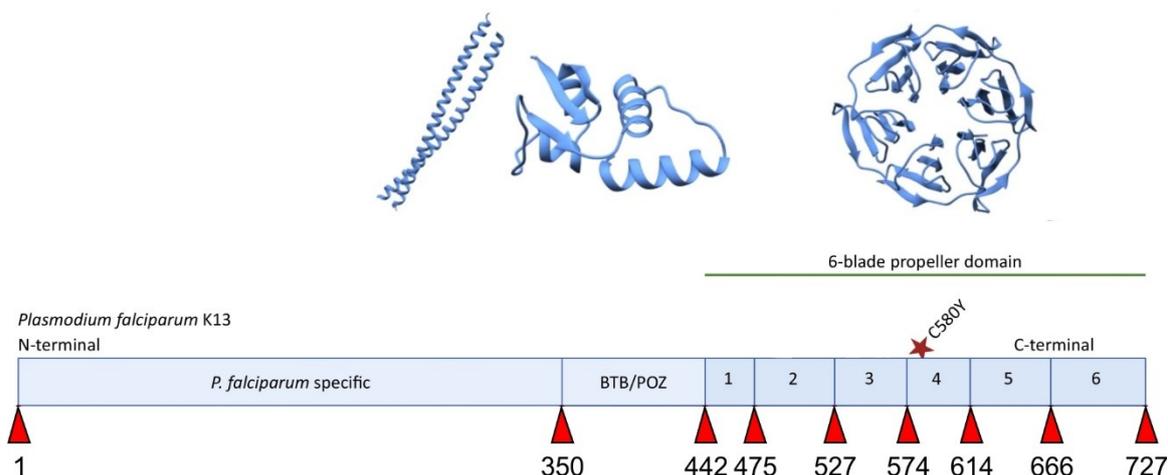
Figure 9) Protein structure of *Pf*Kelch13. (Top) Predicted 3D structure of different *Pf*Kelch13 domains. (Bottom) Protein domains of *Pf*Kelch13 consisting of an N-terminal parasite specific domain, a BTB/POZ domain and a C-terminal Kelch propeller domain. Red triangles indicate amino acid position. Position of ART resistance conferring mutation (C580Y) in the Kelch propeller domain is indicated. (modified from (Tilley *et al.*, 2016; Coppée *et al.*, 2019))

In order to measure ART resistance *in vitro*, the RSA is used to determine the *in vitro* survival rate of young ring stage parasites treated with a pharmacologically relevant ART pulse (dihydroartemisinin (DHA); 700 nM) for 6 hours (Figure 10). After removal of DHA and re-cultivation, the proportion of parasites that survived the drug exposure compared to the untreated control is determined by light microscopy of Giemsa-stained smears after 72 hours (Witkowski & Amaratunga *et al.*, 2013). The decreased *in vitro* drug sensitivity of young ring stages measured by RSA correlates with the observation of delayed parasite clearance in patients and a survival rate of 1% or higher is considered as clinically relevant resistance (Witkowski & Amaratunga *et al.*, 2013).
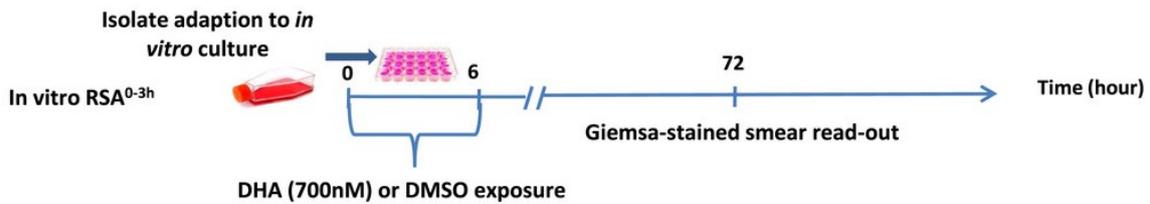
Figure 10) Experimental procedure of standard *in vitro* RSA. Young ring stage parasites are treated with 700 nM DHA for 6 hours. After DHA pulse, the drug is removed, and parasites are re-cultivated for another 66 hours. To assess the proportion of surviving parasites compared to the untreated culture, Giemsa smears are prepared 66 hours post DHA removal. (modified from (Niaré *et al.*, 2018)).

In general, a mutation is validated as molecular marker for ART resistance when this variation confers *in vitro* resistance in standard RSA and reduces the parasite clearance rate after treatment with an ACT in the patient (Witkowski & Amaratunga *et al.*, 2013; WHO, 2017b). The most dominant non-synonymous mutation present in Southeast Asia is the *Pf*Kelch13 C580Y mutation (Ariey *et al.*, 2014). Interestingly, this mutation was also found in Guyana and Papua New Guinea, as well as in Africa where it was not associated with delayed parasite clearance after ACT treatment and displayed only a low prevalence level (< 5%) (Maiga *et al.*, 2012; Ouattara *et al.*, 2015; Chenet *et al.*, 2016; Miotto *et al.*, 2020). The most dominant *pfkelch13* variation in Africa is the SNP resulting in an A578S amino acid change that is present in at least 22 African countries and emerged independently in the different regions (Ménard *et al.*, 2016). This mutation was not associated with a reduced ART-sensitivity (WWARN K13 Genotype-Phenotype Study Group, 2019).

Over the years several different *pfkelch13* SNPs were detected worldwide, and a meta-analysis published in 2019 validated 20 mutations as potential molecular markers for ART resistance (WWARN K13 Genotype-Phenotype Study Group, 2019). Out of those, multiple *pfkelch13* polymorphisms from Southeast Asia were found to confer ART resistance. In contrast, most of the mutations detected in African field isolates do not change the sensitivity to ART and genetic analysis of the corresponding parasites showed only minor local selection of *pfkelch13* mutations (general prevalence lower than 5%) (Kamau *et al.*, 2015; Taylor *et al.*, 2015; MalariaGEN Plasmodium falciparum Community Project, 2016; Ménard *et al.*, 2016). Two recently published studies demonstrated the emergence of ART resistance in Rwanda, validating the *pfkelch13* mutation R561H as molecular marker associated with reduced *in vivo* parasite clearance (Uwimana *et al.*, 2020; Uwimana *et al.*, 2021).

1.4.2.2 *Pf*Kelch13 independent mutations contributing to ART resistance

Beside non-synonymous *pfkelch13* mutations, allelic variations in other candidate genes are suspected to play a role in ART resistance. Data from genotyping assays and Sanger sequencing of culture-adapted Cambodian clinical *P. falciparum* isolates indicated that parasites lacking *pfkelch13* mutations can also be less susceptible to ART as indicated by RSA (Mukherjee *et al.*, 2017).

In the murine parasite strain *P. chabaudi*, mutations in an orthologue of the *P. falciparum* ubiquitin-specific protease-1 (*pfubp1)* were shown to mediate ART resistance (Hunt *et al.*, 2007; Hunt *et al.*, 2010b). The *pfubp1* mutations, V2697F and V2728F, were also tested in *P. falciparum*, as well as *P. berghei*, and rendered parasites resistant to ART in both cases (Henrici *et al.*, 2019; Simwela *et al.*, 2020). Interestingly, one of the allelic variations (V2728F) also led to a reduced sensitivity against chloroquine, mefloquine and lumefantrine (Hunt *et al.*, 2007; Hunt *et al.*, 2010b; Borges *et al.*, 2011; Kinga Modrzynska *et al.*, 2012). Genome wide association studies (GWAS) performed in coastal Kenya also implicate *pfubp1* in ART resistance, as did an analysis of field isolates from Northwestern Thailand which resulted in the identification of different candidate mutations in *pfubp1* that may be involved in reduced ART susceptibility (Borrmann *et al.*, 2013; Cerqueira *et al.*, 2017). In another Kenyan study, *P. falciparum* field isolate samples were obtained from children suffering from malaria and displayed delayed parasite clearance after ACT treatment. These samples were genetically analyzed and also there *pfubp1* mutations were detected, further increasing the evidence for a potential involvement of this candidate gene in ART resistance (Henriques *et al.*, 2014).

In addition to mutations in *pfubp1*, variant alleles of the AP2 adaptor complex µ-chain *(pfap2µ)* were detected in the study conducted in Kenya (Henriques *et al.*, 2014). Initially, this candidate was suggested as a genetic marker for ART resistance in the *P. chabaudi* rodent malaria model (Henriques *et al.*, 2013). The SNP, resulting in the non-synonymous amino acid change I568T, in AP2µ was found to be associated with delayed parasite clearance under laboratory conditions in *P. chabaudi* and this was also confirmed in *P. falciparum* but was not detected in parasites from infected individuals in the field (Henriques *et al.*, 2013; Henriques *et al.*, 2014; Henrici *et al.*, 2019). Another mutation in *Pf*AP-2µ (S160N/T) was identified in field isolates obtained from Kenyan children treated with ACTs and found to reduce sensitivity towards ART (Henriques *et al.*, 2014). This mutation was also shown to simultaneously reduce the susceptibility to quinine, chloroquine and lumefantrine (Henriques *et al.*, 2015).

In both cases, *pfubp1* and *pfap2μ*, parasite susceptibility to ART was reduced, while at the same time the sensitivity to potential partner drugs like mefloquine or lumefantrine was also changed.

A further gene implicated in ART resistance is the *Plasmodium* protein coronin. This protein is important for parasite motility by modulating the actin cytoskeleton (Olshina *et al.*, 2015; Bane *et al.*, 2016). In a long-term *in vitro* selection process with increasing concentrations of ART with a West African parasite strain, three *pf*coronin mutations (G50E / R100K / E107V) were identified and validated by introducing them into the parental strain using CRISPR/Cas9. These variant alleles were then shown to reduce parasite susceptibility to ART by RSA (Demas *et al.*, 2018). In other studies, further mutations were identified *in vitro*, but none the coronin mutations have so far been found to be present in parasites from the field (Sharma *et al.*, 2019; Velavan *et al.*, 2019; Sharma *et al.*, 2020).

Other candidates that are potential markers for ART resistance are *pfeps15* (also annotated as *pfformin2*) and *pfmyosinF* (previously annotated as *pfmyosinC*). These candidates were identified by whole genome sequencing of patient samples collected in Northwestern Thailand. Analysis of long-term changes in allele frequencies revealed two mutations (*Pf*MyosinF: N277S; *Pf*Eps15: K447R) that might be involved in ART resistance, but these mutations were so far not specifically tested *in vitro* for their ability to modulate ART sensitivity (Cerqueira *et al.*, 2017).

1.4.3   Mechanism of ART resistance

The molecular mechanism of ART resistance is widely debated. So far, it had been unclear how *Pf*Kelch13 is involved in ART resistance (Dogovski *et al.*, 2015; Mbengue *et al.*, 2015; Mok *et al.*, 2015; Rocamora *et al.*, 2018).

One hypothesis suggests the upregulation of the unfolded protein response (UPR) as possible mechanism rendering parasites resistant to ART (Mok *et al.*, 2015). These authors analyzed the transcriptional profile of ART resistant isolates. Besides a delay of the intraerythrocytic development in young ring stages, parasites showed an upregulation of two major chaperon complexes that are involved in the UPR pathway (Mok *et al.*, 2015). Two other studies support the finding of an enhanced cellular stress response in ART resistance. Kinetic analysis and comparison of Cambodian wildtype and mutant *Pf*Kelch13 isolates revealed and ART induced growth delay in these parasites and showed an increase of ubiquitinated proteins, suggesting and upregulation of the cellular stress response and

activation of the ubiquitin-mediated proteasomal degradation system in ART treated parasites (Dogovski *et al.*, 2015). A second study investigated the transcriptome of ART resistant isogenic 3D7 *P. falciparum* lineages obtained by long-term ART exposure (Rocamora *et al.*, 2018). These parasites did not contain *Pf*Kelch13 mutations but were moderately resistant to ART and were proposed to achieve this through an increased capacity to handle oxidative stress during ART treatment. Such a function in resistance would be in line with the role of the closest homologues of *Pf*Kelch13 in other systems, e.g. the human Kelch-like proteins (KLHLs) that function as adapters to E3 ubiquitin ligases (Adams *et al.*, 2000; Furukawa *et al.*, 2003; Zhang *et al.*, 2004; Ariey *et al.*, 2014). However, the variety of substrates of KLHLs and their function is very broad (Lee & Yuan *et al.*, 2010; Tseng & Bixby, 2011; Dhanoa *et al.*, 2013; Shi *et al.*, 2019). Hence, the potential substrate regulated by *Pf*Kelch13 cannot be predicted based on the sequence similarity to KLHLs alone. This link to the cellular stress response based on sequence similarity of *Pf*Kelch13 to KLHLs (Ariey *et al.*, 2014; Tilley *et al.*, 2016) is therefore rather tentative.

In the same year, as *Mok et al* and *Dogovski et al* postulated an upregulated unfolded protein response and enhanced cellular stress response as potential mechanisms of ART resistance, *Mbengue et al* provided data that indicated an increased level of phosphatidylinositol-3-phosphate (PI3P) to mediate ART resistance (Mbengue *et al.*, 2015). In this model, PI3P is generated by the phosphatidylinositol-3-kinase (PI3K) that was by this work indicated as a binding partner for wildtype *Pf*Kelch13. At the same time, PI3K is also target of the activated ART. *Pf*Kelch13-PI3K binding regulates the amount of PI3P and parasite growth. In contrast, mutant *Pf*Kelch13 is unable to bind PI3K which leads to an increased PI3P level in the parasite. This higher PI3P level prepares the parasite to counteract the inhibition of PI3K through ART and survive the drug treatment.

In a recently published paper, *Pf*Kelch13 was found to be important for the endocytosis of host cell hemoglobin by the parasite and this was investigated as a potential mechanism to regulate ART resistance (Birnbaum *et al.*, 2020). In a prior study, chemical inhibition of the hemoglobinases (falcipains) as well as genetic knockdown and knockout strategies indicated an importance of hemoglobin digestion for the resistance mechanism (Xie *et al.*, 2016). Another study, analyzing the proteomic, peptidomic and metabolic profiles of ART resistant field isolates revealed that in resistant strains the abundance of hemoglobin-derived peptides was reduced (Siddiqui *et al.*, 2017). *Birnbaum et al* defined a new intracellular compartment by identifying interacting partners of the *Pf*Kelch13 protein (Birnbaum *et al.*, 2020). In a proximity biotinylation approach combined with quantitative mass spectrometry, several proteins were identified that were found to locate in foci in the cell together with Kelch13. This included homologues of proteins that in other organisms

are involved in endocytosis (e.g. *Pf*Eps15, *Pf*AP-2μ), or had previously been suspected in ART resistance, e.g. *Pf*UBP1 (Tebar *et al.*, 1996; Hunt *et al.*, 2007; McMahon & Boucrot, 2011; Cerqueira *et al.*, 2017; Kadlecova *et al.*, 2017; Kaksonen & Roux, 2018). Besides these candidates, several other proteins were detected as potential interacting partners of *Pf*Kelch13, such as *Pf*MyosinF and the *Plasmodium* metacaspase-2 (*Pf*MCA2) (Birnbaum *et al.*, 2020). However, so far, it is not known if the latter two proteins indeed co-locate with *Pf*Kelch13, and the function of these proteins remains unclear. Initial work already showed that parasites with a disrupted *pfmca2* gene displayed a reduced sensitivity to ART (Birnbaum *et al.*, 2020).

1.5 Aims of the present thesis

Previous studies indicated that besides *Pf*Kelch13, other factors contribute to ART resistance (Hunt *et al.*, 2007; Borrmann *et al.*, 2013; Henriques *et al.*, 2013; Cerqueira *et al.*, 2017; Mukherjee *et al.*, 2017; Demas *et al.*, 2018). Using a novel version of BioID and quantitative mass spectrometry, a compartment containing various potential interaction partners of *Pf*Kelch13 was identified (Birnbaum *et al.*, 2020). This analysis resulted in a list of high confidence hits which were mostly of unknown function and were named KICs (Kelch interacting candidates). Interestingly, this list also included proteins that had previously been suspected in ART resistance, such as *Pf*UBP1 or *Pf*Eps15, but also several proteins - e.g. *Pf*MCA2 and *Pf*MyosinF - that had remained functionally uncharacterized in *P. falciparum* so far.

The aim of the thesis was to gain more insight in the newly defined Kelch13 compartment and its proteins and their role in resistance. In particular the aim was to analyze the potential *Pf*Kelch13 interacting candidates *Pf*MCA2 and *Pf*MyosinF in detail since they remain uncharacterized in *P. falciparum* to date. Moreover, a further goal was to assess whether different non-synonymous mutations in *Pf*Kelch13 and in the potential *Pf*Kelch13 compartment members mediate ART resistance in *P. falciparum*. Due to the prevalence of a few well defined *Pf*Kelch13 mutations associated with resistance in Southeast Asia and the scarcity of such mutations in Africa, an additional focus of this thesis was non-synonymous SNPs that are mainly prevalent in Africa. The final aim was to investigate the resistance phenotype of ART resistant *Pf*Kelch13 parasites and to assess the influence of specific mutations on parasite fitness.

2 Material

2.1 Antibodies

2.1.1 Primary antibodies

Antigen	Dilution and application	Source
α-aldolase (rabbit)	1:2000 for WB	
α-GFP (mouse)	1:2000 for WB	Roche, Mannheim
α-HA (rabbit)	1:500 for IFA	
α-HA (rat)	1:500 for IFA 1:1000 for WB	Roche, Mannheim
α-RFP (rat)	1:500 for IFA	Chromotek, München

2.1.2 Secondary antibodies

Antigen	Dilution and application	Source
α-rabbit HRP	1:2500 for WB	Molecular probes
α-rabbit 488	1:2000 for IFA	Molecular probes
α-mouse HRP	1:3000 for WB	Dianova
α-rat HRP	1:3000 for WB	Dianova
α-rat Alexa 594	1:2000 for IFA	Molecular probes

2.2 Bacteria and *Plasmodium* strains

Escherichia coli (XL-10 Gold)   Tet^r Δ(*mcrA*) 183 Δ(*mcrCB-hsdSMRmrr*) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte* [F' *proAB lacI^q Z ΔM15 Tn10* (Tet^r) Amy Cam^r]

Plasmodium falciparum (3D7)   Clone of NF54 isolate (MRA-1000) from a malaria patient near Amsterdam airport (Walliker *et al.*, 1987)

2.3 Chemicals

Acrylamide solution (30%)	AppliChem GmbH, Darmstadt
Acetone	Roth, Karlsruhe
Agar LB (Lennox)	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Albumax II	Gibco, Life Technologies, USA
Albumin bovine Fraction V (BSA)	Biomol, Hamburg
Ammonium persulfate (APS)	AppliChem GmbH, Darmstadt
Ampicillin	Roche, Mannheim
Bacto^{TM} Pepton	Becton Dickinson, Heidelberg
BactoTM yeast extract	Becton Dickinson, Heidelberg
ß-Mercaptoethanol	Merck, Darmstadt
Blasticidin S	Invitrogen
Bromphenol blue	Roth, Karlsruhe
Concovalin A	Sigma Aldrich, Steinheim
Cytochalasin D	Calbiochem, Darmstadt
Desoxynucleotides (dNTPs)	Thermo Scientific, Lithuania
Developer solution G150 (Western Blot)	Agfa, Leverkusen
4´,6-diamidino-2-phenylindole (DAPI)	Roche, Mannheim
Dihydroartemisinin (DHA)	AdipoGen Life Sciences
Dihydroethidium (DHE)	Cayman, Ann Arbor, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
Dipotassium phosphate	Roth, Karlsruhe
Disodium phosphate	Roth, Karlsruhe
1,4-dithiothreitol (DTT)	Roche, Mannheim
DSM1	BEI resources
Dulbecco´s Phosphate Buffered Saline	PAN Biotech, Aidenbach
Ethanol	Roth, Karlsruhe
Ethidium bromide	Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Biomol, Hamburg
Ethylene glycol tetraacetic acid (EGTA)	Biomol, Hamburg
Formaldehyde (10%)	Polyscience, Warrington PA, USA
G418 disulfate salt	Sigma Aldrich, Steinheim
Gentamycin	Ratiopharm, Ulm

Giemsa´s azure, eosin, methylene blue solution	Merck, Darmstadt
D-Glucose	Merck, Darmstadt
Glutardialdehyd (25%)	Roth, Karlsruhe
Glycerol	Merck, Darmstadt
Glycine	Biomol, Hamburg
Hoechst33342	Cheomdex, Switzerland
(4-(2-Hydroxyethyl)-1-piperazineethanesulfonicacid) (HEPES)	Roche, Mannheim
Hydrochloric acid (HCl)	Merck, Darmstadt
Hypoxanthin	Biomol, Hamburg
Isopropanol	Roth, Karlsruhe
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt
Manganese (II) chloride ($MnCl_2$)	Merck, Darmstadt
Methanol	Roth, Karlsruhe
Milk powder	Roth, Karlsruhe
3-(N-morpholino)propansulfonic acid (MOPS)	Sigma Aldrich, Steinheim
N, N, N, N-Tetramethylethylenediamin (TEMED)	Merck, Darmstadt
Percoll	GE Healthcare, Sweden
Phenylmethylsulfonylfluorid (PMSF)	Sigma Aldrich, Steinheim
Potassium chloride	Merck, Darmstadt
Protease inhibitor cocktail ("Complete Mini")	Roche, Mannheim
Rapalog (A/C Heterodimerizer AP21967)	Clontech, Mountain View, USA
Rubidium chloride	Sigma Aldrich, Steinheim
RPMI (Roswell Park Memorial Institute)-Medium	AppliChem, Darmstadt
Saponin	Sigma Aldrich, Steinheim
Sodium acetate	Merck, Darmstadt
Sodium chloride	Gerbu, Gaiberg
Sodium bicarbonate	Sigma Aldrich, Steinheim
Sodium dodecyl sulfate (SDS)	AppliChem, Darmstadt
Sodium dihydrogen phosphate	Roth, Karlsruhe
Sodium hydroxide	Merck, Darmstadt
Sorbitol	Sigma Aldrich, Steinheim
Tris base	Roth, Karlsruhe
Tris-EDTA (TE)	Invitrogen, Karlsruhe
Triton X-100	Biomol, Hamburg
Water for molecular biology (Ampuwa)	Fresenius Kabi, Bad Homburg

WR99210   Jacobus Pharmaceuticals,
                                                 Washington, USA

2.4 DNA and protein ladders

GeneRuler™ 1000 bp ladder   Thermo Scientific, Schwerte
PageRuler™ Plus Prestained Protein Ladder   Thermo Scientific, Schwerte
(10 to 250 kDa)
PageRuler™ Prestained Protein Ladder   Thermo Scientific, Schwerte
(10 to 180 kDa)

2.5 Enzymes and polymerases

2.5.1 Restriction endonucleases

AvrII   New England Biolabs, Ipswich
BamHI-HF®   New England Biolabs, Ipswich
DpnI   New England Biolabs, Ipswich
KpnI- HF®   New England Biolabs, Ipswich
MluI- HF®   New England Biolabs, Ipswich
NotI- HF®   New England Biolabs, Ipswich
SalI- HF®   New England Biolabs, Ipswich
XhoI   New England Biolabs, Ipswich

2.5.2 Polymerases

FirePol DNA Polymerase [5 U/µl]   Solis Biodyne, Taipei
Phusion® High-Fidelity DNA Polymerase [2 U/µl]   New England Biolabs, Ipswich

2.5.3 Oligonucleotides

All oligonucleotides are listed in Appendix C   Sigma Aldrich, Steinheim

2.6 Kits

NucleoSpin. Extract II	Macherey-Nagel, Düren
NucleoSpin. Plasmid	Macherey-Nagel, Düren
QIAamp DNA Mini Kit	Qiagen, Hilden
QIAGEN Plasmid Midi Kit	Qiagen, Hilden
Western Blot ECL-Clarity Detection Kit	Bio-Rad, München
Western Blot ECL-SuperSignal West Pico	Thermo Scientific, Schwerte

2.7 Labware and disposables

Type	Specification	Distributor
Conical falcon tubes	15 ml, 50 ml	Sarstedt, Nümbrecht
Cryotubes	1.6 ml	Sarstedt, Nümbrecht
Culture bottles	50 ml	Sarstedt, Nümbrecht
Disposable pipette tips	1-10/20-200/100-1000 µl	Sarstedt, Nümbrecht
Filter tips	1-10/20-200/100-1000 µl	Sarstedt, Nümbrecht
Flow cytometry tubes	55.1579	Sarstedt, Nümbrecht
Glass cover slips	24x55 mm thickness 0.13-0.16 mm	R. Langenbrinck, Emmerdingen
Glass slides		Engelbrecht, Edermünde
Gloves, latex powder-free		Kimtech Science EcoShield
IFA glass slides	10 wells ER-208B-CE24; 6.7 mm	Thermo Scientific, USA
Leukosilk tape		BSN medical
Microscopy dishes, uncoated, hydrophobic		Ibidi, Martinsried
Multiply-µStrip Pro 8-Strip PCR reaction tubes		Sarstedt, Nümbrecht
Nitrocellulose blotting membrane Protean	Amersham 0.45 µm	GE Healthcare
One-way canulas		Braun, Melsungen
One-way syringe		Braun, Melsungen
Parafilm		Bemis, USA

Pasteur pipettes		Brand, Wertheim
Petri dishes	15x60 mm/14x90 mm	Sarstedt, Nümbrecht
Plastic pipettes	5/10/25 ml	Sarstedt, Nümbrecht
Reaction tubes	1.5/2 ml	Sarstedt, Nümbrecht Eppendorf, Hamburg
Scalpel		Braun, Melsungen
Sterile filter	0.22 µm	Sarstedt, Nümbrecht
Transfection cuvettes	0.2 cm	Bio-Rad, München
12-well cell culture plate		Sarstedt, Nümbrecht

2.8 Plasmids

Plasmid	Source
pSLI-3xHA (pARL1-3xHA-T2A-Neo[R])	Mesen-Ramirez
pSLI-TGD (pARL1-GFP-T2A-Neo[R])	Birnbaum Nat Comm 2017
pSLI-N-GFP-2xFKBP-loxP (DSM1[R])	Birnbaum Nat Comm 2017
nmd3 5`UTR_mCherry-Kelch13 (DSM1[R])	Birnbaum Nat Comm 2017
nmd3 5`UTR_chromobody (DSM1[R])	Sabitzki
crt 5`UTR_1xNLS-FRB-T2A-P40-mCherry (DSM1[R])	Flemming

2.9 Solutions, media, and buffer

2.9.1 Microbiological culture

10x LB stock solution	10% NaCl 5% peptone 10% yeast extract in dH_2O, autoclaved
LB medium	1% (w/v) NaCl 0.5% (w/v) peptone 1% (w/v) yeast extract in dH_2O in dH_2O
LB Agar plate solution	1.5% Agar-Agar 1x LB medium
Ampicillin stock solution	100 mg/ml in 70% ethanol

Glycerol freezing solution		50% (v/v) glycerol in 1x LB medium
TFBI buffer		30 mM acetic acid 50 nM MnCl$_2$ 100 mM RbCl 10 mM CaCl$_2$ 15% (v/v) glycerol pH 5.8 (with 0.2 N acetic acid)
TFBII buffer		10 mM MOPS 75 mM CaCl$_2$ 10 mM RbCl 15% (v/v) glycerol pH 7.0 (with NaOH)

2.9.2 Molecular biological buffers and solutions

DNA gel electrophoresis	50x TAE	2 M Tris base 1 M Pure acetic acid 0.05 M EDTA pH 8.5
	6x Loading buffer	40% Glycerol (v/v) 2.5% (w/v) Xylene cyanol 2.5% (w/v) Bromophenol blue in dH$_2$O
DNA precipitation	Sodium acetate	3 M NaAc, pH 5.2
	Tris-EDTA (TE) buffer	10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0
Gibson assembly buffers	5x isothermal reaction buffer	3 ml 1 M Tris-HCl pH 7.5 150 µl 2 M MgCl$_2$ 60 µl each of 100 mM dGTP/dATP/dTTP/dCTP 300 µl 1 M DTT 1.5 g PEG-8000 300 µl 100 nM NAD ad 6 ml dH$_2$O
	Assembly master mixture (1.2 ml)	320 µl 5x isothermal reaction buffer 0.64 µl 10 U/µl T5 exonuclease 20 µl 2 U/µl Phusion DNA polymerase 160 µl 40 U/µl Taq DNA ligase

ad 1.2 ml dH$_2$O

2.9.3 Biochemical buffers and solutions

10x Running buffer	29 g Tris base (24 mM) 144 g Glycine (192 mM) 10 g SDS (3.5 mM)
Separating gel buffer (buffer A)	1.5 M Tris 0.4% SDS pH 8.8
Stacking gel buffer (buffer B)	0.5 M Tris 0.4% SDS pH 6.8
Separating gel (10%) (∑ 10 ml; 2 gels)	4 ml dH$_2$O 2.7 ml 1.5 M Tris pH 8.8 (buffer A) 3.3 ml 30% acrylamide 6 µl TEMED 100 µl 10% APS
Separating gel (8%) (∑ 10 ml; 2 gels)	4.8 ml dH$_2$O 2.5 ml 1.5 M Tris pH 8.8 (buffer A) 2.7 ml 30% acrylamide 6 µl TEMED 100 µl 10% APS
Separating gel (6%) (∑ 10 ml; 2 gels)	5.3 ml dH$_2$O 2.7 ml 1.5 M Tris pH 8.8 (buffer A) 2 ml 30% acrylamide 6 µl TEMED 100 µl 10% APS
Stacking gel (∑ 5 ml; 2 gels)	2.9 ml dH$_2$O 1.25 ml 0.5 M Tris pH 6.8 (buffer B) 830 µl 30% acrylamide 5 µl TEMED 50 µl 10% APS
Ammonium persulfate (APS)	10% (w/v) in dH$_2$O
4x SDS sample buffer	3 ml 0.5 M Tris pH 6.8 2 ml dH$_2$O 5 g sucrose 0.32 g SDS
10x Transfer Buffer	30.3 g Tris base 144.1 g glycine 3.7 g SDS

| 1x Transfer buffer + 20% methanol | 1:10 dilution of 10x Transfer Buffer
20% methanol |

2.9.4   Cell biological buffers, media, and solutions (*P. falciparum in vitro* culture)

RPMI complete medium	1.587% (w/v) RPMI 1640 12 mM NaHCO$_3$ 6 mM D-Glucose 0.5% (v/v) Albumax II 0.2 mM Hypoxanthine 0.4 mM Gentamycin pH 7.2 in dH$_2$O, sterile filtered
Synchronization solution	5% (w/v) D-Sorbitol in dH$_2$O, sterile filtered
Parasite freezing solution	4.2% (w/v) D-Sorbitol 0.9% (w/v) NaCl 28% (v/v) Glycerol in dH$_2$O, sterile filtered
Parasite thawing solution	3.5% (w/v) NaCl in dH$_2$O, sterile filtered
Transfection buffer (Cytomix)	120 mM KCl 150 µM CaCl$_2$ 2 mM EGTA 5 mM MgCl$_2$ 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ 25 mM HEPES pH 7.6 in dH$_2$O, sterile filtered
Amaxa transfection buffer	90 mM NaPO$_4$ 5 mM KCl 0.15 mM CaCl$_2$ 50 mM HEPES pH 7.3 in dH$_2$O, sterile filtered
WR99210 stock solution	20 mM WR99210 in DMSO
WR99210 working solution	1:1000 dilution of stock solution in RPMI complete medium sterile filtered
Blasticidin S (BSD) working solution	5 mg/ml BSD in RPMI complete medium sterile filtered

G418 working solution	50 mg/ml in RPMI complete medium sterile filtered
DSM1 stock solution (50x)	187.5 mM DSM1 in DMSO
DSM1 working solution	100 µl DSM1 stock solution ad 5 ml in 95% DMSO / 5% 1x PBS solution
Human red blood cells	Bloodgroup 0+, sterile concentrate, Blood bank Universitätsklinikum Eppendorf (UKE) Hamburg
Percoll stock solution	90% (v/v) Percoll 10% (v/v) 10x PBS
80% Percoll solution%	89% (v/v) Percoll stock solution 11% (v/v) RPMI complete medium 4% (w/v) sorbitol sterile filtered
60% Percoll solution	67% (v/v) Percoll stock solution 33% (v/v) RPMI complete medium 4% (w/v) sorbitol sterile filtered
40% Percoll solution	44% (v/v) Percoll stock solution 56% (v/v) RPMI complete medium 4% (w/v) sorbitol sterile filtered
Saponin solution for selective membrane permeabilization	0.03% Saponin in DPBS
Parasite lysis buffer	4% SDS 0.5% Triton 0.5x PBS in dH_2O
Cytochalasin D working solution	10 µM Cytochalasin D in DMSO
E64 inhibitor working solution	33 µM in dH_2O
DHA stock solution (250 µM)	1.1 mg DHA powder in 15 ml DMSO
DHA working solution (125 µM)	1:2 dilution of DHA stock solution in 1xPBS/DMSO (1:10)
DHE stock solution (10x)	5 mg DHE in 1 ml DMSO

DHE working solution (1x)   0.5 mg DHE
                                             in 1 ml DMSO

Hoechst33342 stock solution (10x)   4.5 mg Hoechst33342
                                             in 1 ml DMSO

Hoechst33342 working solution (1x)   0.45 mg Hoechst33342
                                             in 1 ml DMSO

FACS stop solution   0.5 µl Glutaraldehyde (25%)
                                             in 40 ml RPMI complete medium

Rapalog (AP21967) stock solution   500 mM Rapalog
                                             in ethanol

Rapalog working solution   1:20 dilution of stock solution in RPMI
                                             complete medium

Giemsa staining solution   1:10 dilution of stock solution in H_2O

Fixing solution   2 ml formaldehyde 10%
(formaldehyde/glutaraldehyde IFA)   1.5 µl glutaraldehyde 25%
                                             500 µl 10x PBS
                                             ad dH_2O to 5 ml total volume

2.10 Technical devices

Type	Specification	Distributor
Agarose gel chamber	Sub Cell GT basic	Bio-Rad, München
Analytical Balance	870	Kern
Blot device Gel holder cassettes Foam pads Electrode assembly Cooling unit	Mini Protean Tetra Cell System	Bio-Rad, München
Centrifuge	Megafuge 1.0R J2 HS Ultracentrifuge Rotor JA-12 Avanti J-26S XP Rotor JA-14	Hereaus, Hannover Beckmann Coulter, Krefeld
Table centrifuge	Eppendorf 5415 D	Eppendorf, Hamburg
Casting gel stuff Casting stand	Mini Protean	Bio-Rad, München

Casting plates Casting frames 12-well combs		
Confocal microscope	Olympus FV1000	Olympus, Hamburg
Developer	ChemiDoc XRS+	Bio-Rad, München
Electrophoresis chamber	Mini Protean 67s	Bio-Rad, München
Electroporator	Gene Pulser X-Cell Nucleofector II AAD-1001N	Bio-Rad, München Amaxa Biosystems, GER
Flow cytometer	LSR II	BD Instruments, USA
Ice machine	EF 156 easy fit	Scotsmann, Venon Hills, USA
Bacterial incubator	Thermo function line	Hereaus, Hannover
P. falciparum cell culture incubator	Heratherm IGS400	Thermo Scientific, Langenselbold
Shaking incubator	Max Q4000	Barnstead, Iowa, USA
Light microscope	Axio Lab A1	Zeiss, Jena
Fluorescence microscope	Axioscope M1/M2	Zeiss, Jena
Hamamatsu digital camera	Orca C4742-95	Hamamatsu Phototonics K.K., Japan
Microwave	Micro 750W	Whirlpool, China
Laboratory scale	Acculab Atilon-ATL	Sartorius, Göttingen
PCR mastercycler	epgradient	Eppendorf, Hamburg
pH meter	SevenEasy	Mettler-Toledo, Gießen
Photometer	NanoDrop	
Pipettes	1-10/200/1000 µl	Gilson, Middleton, USA
Pipettor	Pipetboy acu	IBS, USA
Power supply	EV31 Power Source 300V	Consort, Belgium VWR, Taiwan
Roller mixer	STR6	Stuart
Sterile bench	Steril Gard III Advance Safe 2020	Baker, Stanford, USA Thermo Scientific, Pinneberg
Thermoblock	Thermomixer compact	Eppendorf, Hamburg
Ultrapure water purification system	Milli-Q	Millipore, Bedfort, USA
UV transluminator	PHEROlum289	Biotec Fischer, Reiskirchen

Vacuum pump	BVC Control	Vacuubrand, Deutschland
Vortexer	VF2	Jank & Kunkel IKA Labortechnik
Waterbath	1083	GFL, Burgwedel

2.11 Bioinformatic tools and databases

Bioinformatic tool / database	Source
InterPro	https://www.ebi.ac.uk/interpro/
MalariaGEN	https://www.malariagen.net/
MotifScan	http://myhits.isb-sib.ch/cgi-bin/motif_scan
NCBI databases	https://www.ncbi.nlm.nih.gov/
PlasmoDB	http://plasmodb.org/plasmo/
PubMed	http://www.ncbi.nlm.nih.gov/pubmed
WWARN	https://www.wwarn.org/

2.12 Computer software

A plasmid Editor (ApE)	Open source (https://jorgensen.biology.utah.edu/wayned/ape/)
Axio Vision 40 v4.7.0.0	Zeiss, Jena
Corel DRAW X8 (64-bit)	Corel Corporation, Ottawa
Corel PHOTO-PAINT X8 (64-bit)	Corel Corporation, Ottawa
FlowJo	Becton, Dickinson Company
GraphPad Prism 9	GraphPad Software, La Jolla, USA
ImageJ 1.53e	Wayne Rasband and contributors, NIH, USA
Image Lab v 5.2.1. build 11	Bio-Rad Laboratories,München
Imaris x64 7.8	Bitplane AG, Zürich, Switzerland
Microsoft Office 2020	Microsoft Corporations, Redmond, USA

3 Methods

3.1 Molecular biological methods

3.1.1 Polymerase chain reaction (PCR)

DNA product was amplified from 3D7 gDNA using two different DNA polymerases. The Phusion-high fidelity polymerase (NEB) has a proof-reading function and was mainly used to amplify DNA sequences with high need of accuracy, e.g. cloning of plasmids for parasite transfection. The second polymerase was the FIREPol polymerase (Solis Biodyne) which was used to amplify very long DNA sequences or was used to perform analytical PCRs like colony screens or integration checks, where no proof-reading function is needed. For each PCR amplification, the annealing or elongation time, as well as the respective temperature was adjusted. The basic settings for the PCR amplification and the PCR program are listed below. After PCR amplification, the PCR products were analyzed by gel electrophoresis to check the size of the amplified fragment.

Phusion PCR		FIREPol PCR	
5x Phusion buffer	10 µl	10x FIREPol buffer B	5 µl
dNTP`s	5 µl	dNTP`s	5 µl
Primer fw (1:10 dilution)	1 µl	$MgCl_2$	4 µl
Primer rv (1:10 dilution)	1 µl	Primer fw (1:10 dilution)	2.5 µl
Phusion DNA polymerase	0.3 µl	Primer rv (1:10 dilution)	2.5 µl
Template	0.3 µl	FIREPol DNA polymerase	0.25 µl
dH_2O	32.4 µl	Template	0.3 µl
		dH_2O	30.75 µl

PCR program (Phusion)				PCR program (FIREPol)		
Denaturation	94°C	2 min	1 cycle	Denaturation	95°C	3 min
Denaturation	94°C	20 sec		Denaturation	95°C	40 sec
Annealing	43°C	30 sec	30 cycles	Annealing	43°C	45 sec
Elongation	63°C	*		Elongation	63°C	*
	72°C	4 min				

* Time was adapted to the size of fragment.

3.1.2 Agarose gel electrophoresis (Garoff & Ansorge, 1981)

To check the size of the DNA fragments either amplified by PCR or digested vector, a 1% agarose gel in 1xTAE buffer containing 1 µg/ml ethidium bromide was used. DNA fragments are separated in an electric field according to their length and negatively charged backbone. The agarose gel was prepared by dissolving the agarose (Invitrogen) in 1x TAE buffer to a final concentration of 1%. To melt the agarose, the solution was carefully heated in a microwave until the total amount of agarose was dissolved. The agarose solution was cooled, and ethidium bromide was added to a final concentration of 1 µg/ml. The agarose gel was poured into a gel tray, a 26-slot comb was added, and gel was cooled until it was completely polymerized. The solid agarose gel was then placed into the electrophoresis chamber, which was filled with 1x TAE buffer, covering the whole gel with liquid. DNA samples were diluted in 6x loading dye and were pipetted into the slots. Additionally, DNA ladder was added to check the size of the DNA fragment. Electrophoretic separation was performed for 25 – 30 min at 150 V. After separation, agarose gel was analyzed using the Chemidoc XRS+.

3.1.3 Clean-up of PCR product or digested plasmids

Either the PCR product, or digested plasmid fragments were purified according to manufacturer protocol using the NucleoSpin Extract II Kit (Macherey-Nagel). The purified PCR product, or fragmented plasmid was eluted in 30 µl elution buffer (buffer AE) and stored at -20°C.

3.1.4 Enzymatic digestion of PCR products and plasmids

Enzymatic digestion using different endonucleases was used for three different scenarios: For the cloning process, the vectors were digested with suitable restriction endonucleases to create sticky ends which enable ligation process. Moreover, to deplete residual methylated DNA, PCR products amplified from plasmid template (MINI or MIDI) were digested with the endonuclease DpnI for 1 hour at 37°C. In addition, restriction digest was

used to perform an analytical digest of MINI preparation product to exclude recombination events and check the correct size of the plasmid. Master mix for enzymatic digest is listed below. Both, the cloning vector, as well as the test digestion mix were incubated for 2.5 hours at 37°C.

Digestion for cloning		Test digestion	
10x CutSmart® Buffer (NEB)	5 µl	10x CutSmart® Buffer (NEB)	1 µl
Restriction enzyme	1 µl	Restriction enzyme	0.2 µl
Template (PCR product/vector)	10 µl	Template (MINI/vector)	2 µl
dH$_2$O	ad 50 µl	dH$_2$O	ad 10 µl

3.1.5   DNA ligation by Gibson assembly

DNA ligation via Gibson assembly is a one-step isothermal DNA assembly that enables the simultaneous ligation of 6 different inserts at a time (Gibson *et al.*, 2009). For an efficient ligation, no sticky ends but overlapping homologous regions at both ends of the insert of 15 – 35 bp are needed. When a PCR product was amplified from plasmid DNA, PCR product was digested with DpnI prior to ligation to deplete methylated DNA. The ligation mix is listed below, and Gibson mix was incubated at 50°C for 1 hour.

Gibson assembly	
Gibson mix	7.5 µl
pre-digested vector DNA	1 µl
PCR product	0.5 µl
dH$_2$O	ad 10 µl

3.1.6   Colony-PCR to screen bacterial clones

After transformation (see 3.2.2), the bacterial clones were screened by PCR to detect single clones that carry the correct plasmid. For this, 10 µl FIREPol PCR mix per tested clone were prepared, using an insert specific and a plasmid specific primer to detect the correct clones. The corresponding PCR mix is listed below. A single clone was picked using a pipette tip, plated onto a fresh pre-warmed LB ampicillin plate, and inoculated in 10 µl prepared PCR

mix. Per construct at least 10 single clones were screened. After PCR amplification, the size of the products controlled by agarose gel electrophoresis (see 3.1.2).

FIREPol PCR	
10x FIREPol buffer B	5 µl
dNTP`s	5 µl
MgCl$_2$	4 µl
Primer fw	2.5 µl
Primer rv	2.5 µl
FIREPol DNA polymerase	0.25 µl
Template	0.3 µl
dH$_2$O	30.75 µl

PCR program (FIREPol)			
1 cycle	Denaturation	95°C	3 min
25 cycles	Denaturation	95°C	40 sec
	Annealing	43°C	45 sec
	Elongation	63°C	*

* Time was adapted to the size of fragment.

3.1.7   Sequencing of plasmids

To exclude recombination events or mutations, the plasmid isolated from a single bacterial clone was sent to Seqlab for sequencing. For this, ~40 ng/µl MINI preparation was mixed with 2 µl premixed primer specific to the desired genomic sequence and adjusted with dH$_2$O to a final volume of 15 µl in an Eppendorf tube. The sequencing samples were then sent to Seqlab, Göttingen for sequence check.

3.1.8   DNA precipitation

After MIDI preparation, DNA was diluted in 200 µl TE buffer and DNA concentration was determined by spectrometry. For transfection of *P. falciparum* either 50 µg (merozoite

transfection) or 100 µg (ring transfection) DNA were needed. The respective MIDI volume containing 50 / 100 µg DNA was mixed with 1:10 volume sodium acetate 3 M, pH 5.0 and precipitated with three volumes of absolute ethanol. After this step, the precipitation mix was centrifuged at 16 000 g for 10 min and supernatant was carefully discarded. The pellet was washed once using 50 µl ethanol 70% and the mix was centrifuged again at 16 000 g for 5 min. After removal of the supernatant, the DNA pellet was air-dried under sterile conditions. For ring transfection, the air-dried DNA pellet was dissolved in 15 µl TE buffer and 385 µl cytomix. For schizont transfection, the DNA pellet was resuspended in 10 µl TE buffer and 90 µl Amaxa transfection buffer. The precipitated DNA was stored at -20°C until transfection.

3.1.9   Isolation of *P. falciparum* genomic DNA for integration check

To check the correct integration of a plasmid into the endogenous locus of *P. falciparum*, genomic DNA (gDNA) was isolated from the parasite. For this, 5 -10 ml of *P. falciparum* cell culture were harvested containing a high trophozoite parasitemia. The parasite culture was pelleted at 1800 rpm for 5 min and supernatant was discarded. To store the parasite pellet and continue the gDNA isolation at a later time, the pellet was mixed with 20 µl proteinase K and frozen at -20°C. When the gDNA isolation was directly performed, the pelleted *Plasmodium* culture was processed according to manufacturer`s protocol using the QIAamp DNA Mini Kit. After completion of the protocol, gDNA was dissolved in 200 µl TE buffer and stored at -20°C.

3.1.10 Integration check using PCR

PCR amplification was used to check the correct integration of a construct into *Plasmodium* DNA. For this, genomic DNA was isolated from parasite cell culture (see 3.1.9) and PCR mix was prepared using FIREPol polymerase. For each approach 10 µl total master mix volume was prepared. The master mix and PCR program are listed below. To check the genetic integration, three different primer combinations were used. To confirm correct 5`UTR integration, a forward primer specific for the upstream region of the modified gene locus was combined with a reverse primer specific for the construct. The 3`UTR integration was tested using a forward primer, specific for the construct and a reverse primer, specific for a sequence downstream of the 3`end of the modified locus. To check depletion of original

locus in the modified parasites, the 5` forward primer, specific for the upstream region of the modified locus was combined with the 3`reverse primer, specific for a sequence downstream of the modified locus. After PCR amplification, the size of the DNA fragments was analyzed by agarose gel electrophoresis (see 3.1.2).

FIREPol PCR	
10x FIREPol buffer B	5 µl
dNTP`s	5 µl
$MgCl_2$	4 µl
Primer fw	2.5 µl
Primer rv	2.5 µl
FIREPol DNA polymerase	0.25 µl
Template	0.3 µl
dH_2O	30.75 µl

PCR program (FIREPol)			
1 cycle	Denaturation	95°C	3 min
25 cycles	Denaturation	95°C	40 sec
	Annealing	45°C	45 sec
	Elongation	61.5°C	*

* Time was adapted to the size of fragment.

3.1.11 Sequencing of *P. falciparum* field isolates

During the Fever without source study, conducted at the Presbytarian Hospital of Agogo in Ghana between 2014 and 2015, *Plasmodium* gDNA from field isolates was purified from patient blood samples. To sequence the genome of these field isolates and to check for mutations in different loci, sequence specific primers were designed (see Appendix C). For this, the genomic sequences of the target genes were subdivided into sections of 3000 bp including the respective 5`UTR region and 3`UTR regions. For PCR amplification of the sections, primers for outer and nested PCR were designed. Additionally, primers for sequencing were designed. Here, the 3000 bp sections were subdivided again into fragments of ~300 bp. When PCR product was amplified, the DNA was cleaned up using the NucleoSpin Extract II Kit (Macherey-Nagel) and DNA fragments were sequenced either

in-house or PCR products were sent to Seqlab for sequencing (PCR amplification, in-house sequencing, and preparation of Seqlab performed by Birgit Förster). For Seqlab, ~15 ng/µl PCR product were mixed with 2 µl of the specific sequencing primer and dH$_2$O was added to a final volume of 15 µl. The results of the sequencing were compared to the original genomic sequences to search for potential mutations.

3.2 Microbiological methods

3.2.1 Preparation of chemical competent *E. coli* bacteria (Hanahan, 1983)

To chemically prepare the *E. coli* bacteria for the transformation, the rubidium-chloride method was used, resulting in highly competent cells ready for plasmid uptake. A glycerol stock of XL-10 gold *E. coli* bacteria was inoculated in 20 ml LB medium, and bacteria were grown overnight in a 50 ml falcon. Here, the lid of the 50 ml falcon was not fully closed to supply the bacteria with a sufficient amount of fresh air. The next day, 8 ml of the overnight bacteria culture were given into 200 ml fresh, prewarmed LB medium and the culture was cultivated in a shaker at 750 rpm until the XL-10 gold *E. coli* reached an optical density (OD) of 0.5 to 0.6. When the correct optical density was achieved, the bacteria were cooled down rapidly on ice and centrifuged at 2400 g for 10 to 20 minutes (min) at 4°C. After this step, the bacteria pellet was resuspended in 60 ml TFB I buffer and incubated for 10 min on ice. The bacteria suspension was pelleted again (2400 g for 10 to 20 min at 4°C), supernatant was discarded, and the bacteria pellet was resolved in TFB II buffer. This *E. coli* suspension was then aliquoted in 100 µl aliquots and stored at -80°C.

3.2.2 Transformation of chemical competent XL-10 gold *E. coli*

Before starting the transformation procedure, a fresh LB agar plate (containing ampicillin selection marker) was pre-warmed at 37°C in the incubator and an aliquot of XL-10 gold bacteria was thawed on ice for at least 15 min. For transformation, the total amount of prepared Gibson assembly mix (see 3.1.5) was added to the competent bacteria, mixed carefully, and put on ice for 10 min. In case of a re-transformation, 0.3 µl of diluted plasmid (1:1000 in dH$_2$O) was added to the XL-10 gold suspension and also incubated on ice for 10 min. The ice incubation period was followed by a heatshock at 42°C for 30 seconds (sec) after which, the transformation mix was incubated for another 10 min on ice. After the

second incubation, the bacteria were plated on the pre-warmed LB agar plate containing ampicillin. LB agar plates were incubated overnight at 37°C in the bacteria incubator. Next day, correct bacteria clones were detected via colony PCR screening (see 3.1.6) and a master plate of these clones was prepared and grown overnight at 37°C.

3.2.3 Overnight culture of transformed *E. coli* clone for plasmid purification

A correct, single bacteria clone was detected by colony PCR screen and confirmed by sequencing (see 3.1.7), picked with a pipette tip from the LB agar master plate, and inoculated in fresh LB medium containing the antibiotic ampicillin. For a MINI precipitation, 2 ml LB ampicillin medium were prepared in an Eppendorf tube, while for MIDI precipitation 200 ml LB ampicillin medium were prepared in an Erlenmeyer flask. The MINI bacteria culture was incubated on a thermo shaker at 37°C, 750 rpm overnight and the MIDI bacteria culture was shaken at 37°C and 170 rpm overnight. Next day, the bacteria cultures, MINI or MIDI, were pelleted and either directly purified or stored at -20°C until the next precipitation was performed.

3.2.4 Freezing of *E. coli* culture

For a plasmid-backup, a glycerol stock of the overnight culture containing a single *E. coli* clone was prepared. Here, 500 µl of the overnight culture were pelleted and the bacteria pellet was resuspended in 1 ml LB glycerol medium and stored immediately at -80°C.

3.2.5 Plasmid purification (MINI and MIDI preparation)

To purify ~20 µg plasmid DNA via a MINI preparation, an overnight bacteria culture from a single clone was prepared, as described in section 3.2.3. The 2 ml MINI bacteria culture was pelleted by centrifugation at 11 000 g for 1 min, the supernatant was discarded, and bacteria pellet was processed according to the manufacturer protocol of using the NucleoSpin Plasmid Kit (Macherey-Nagel). After plasmid purification, DNA concentration was measured using the NanoDrop. The correctness of the plasmid was confirmed by a test digestion using the suitable restriction enzymes and the MINI plasmid DNA was sent for sequencing to Seqlab to exclude potential inserted mutations (see 3.1.7).

Higher amounts of plasmid DNA were isolated and prepared for parasite transfection using a MIDI preparation kit. For this, 200 ml overnight bacteria culture of a single, correct bacteria clone was prepared as described in section 3.2.3. The next day, the overnight culture was centrifuged at 6000 rpm, for 15 min at 4°C and the pellet was processed according to the manufacturer protocol using the Plasmid MIDI Kit (QIAGEN). After the plasmid purification, the DNA pellet was dissolved in 200 µl TE buffer and DNA concentration was determined by spectrometry.

3.3 Cell biological methods

3.3.1 Cultivation of *P. falciparum* (Trager & Jensen, 1976)

Continuous cultivation of *P. falciparum* parasites was performed according to standard procedures (Trager & Jensen, 1976). Asexual blood stage parasites were grown at 37°C in 15 x 60 mm Petri dishes (5 ml RPMI complete medium) or 14 x 90 mm Petri dishes (10 ml RPMI complete medium) and atmosphere was adjusted to 1% O_2, 5% CO_2, 94% N_2. Parasites were cultured in RPMI complete medium with 0.5% Albumax, human erythrocytes (blood group 0+) at a hematocrit of 5%. Every second day parasite cultures were smeared, diluted, or fed to keep parasitemia between 0.1% - 5%, and supplied with fresh blood and medium to avoid parasite starvation. In case of high parasitemia, RPMI medium was changed daily. Transgenic parasites were selected using 4 nM WR99210, 2 µg/ml blasticidin S or 0.9 µM DSM1.

3.3.2 Preparation of blood smears and Giemsa staining of parasite cultures

To determine parasitemia during continuous *Plasmodium* culturing, blood smears were taken from the cultures and stained with Giemsa staining solution. Additionally, blood smears were used to determine the proportion of surviving parasites at 72 hours in a standard ring stage survival assay (RSA). For the blood smears, ~0.5 µl parasite culture were taken and dropped on a glass slide. A thin smear was prepared using a second glass slide, and thin blood smear was air-dried. The dry thin smears were fixed in methanol (100%) for 20 sec and stained in Giemsa solution (10% in H_2O) for 15 min. After staining, the glass slides were rinsed with fresh tap water and air-dried. Parasitemia was assessed by light microscopy.

3.3.3 Freezing of *P. falciparum* culture for storage

For storage, parasite cell lines were frozen either at -80°C or in liquid nitrogen. Thus, only ring stage parasites will survive the freezing process, a parasite culture containing high ring parasitemia was prepared for freezing. For cryopreservation, 5 – 10 ml of cell culture were harvested and pelleted at 2000 rpm for 5 min. After centrifugation, the supernatant was removed, and the pellet was resuspended in 1 ml parasite freezing solution. The parasite solution was pipetted into a 2 ml cryotube, labelled and frozen either at -80°C or in liquid nitrogen for long-term storage.

3.3.4 Thawing of *P. falciparum* cryo-stabilates

To re-cultivate a parasite line after cryopreservation, the cryo-stabilates were thawed in a water bath at 37°C for 2 – 3 min. The thawed cell lines were transferred into a 2 ml Eppendorf tube and centrifuged at 3500 rpm for 1.5 min. The supernatant was discarded, and the pellet was carefully dissolved in parasite thawing solution. After another centrifugation step, the pellet was washed in fresh RPMI complete medium and centrifuged again. The parasite pellet was resuspended in 5 ml of fresh RPMI complete medium, placed in a Petri dish and fresh human blood was adjusted to a hematocrit of 5%. For transgenic parasites grown under drug pressure, selective drug was added on the second day after thawing.

3.3.5 Synchronization of asexual parasite culture

The synchronization of parasites is important for different experiments but is also necessary for the preparation of *P. falciparum* parasites for transfection. To synchronize an asexual parasite culture, two different approaches can be used.

Synchronization of parasites to 0 – 18 hours ring stages can be achieved by lysis of mature stages via 5% D-sorbitol (Lambros & Vanderberg, 1979). Here, the parasite culture was transferred to a 15 ml Falcon tube and pelleted at 2000 rpm for 5 min. The parasite pellet was resuspended in pre-warmed D-sorbitol solution (1 ml D-sorbitol for 5 ml culture and 2 ml D-sorbitol for 10 ml culture) and incubated in the water bath at 37°C for 7 min. After incubation, the cell culture was centrifuged at 2000 rpm for 5 min and pellet was washed

twice in fresh RPMI complete medium to remove leftover D-sorbitol. After washing, the parasite pellet was resuspended in fresh RPMI medium and recultivated in a Petri dish.

To synchronize an asexual parasite culture for schizonts, a Percoll gradient was used. Here the different parasite stages were separated due to their differences in permeability (Aley *et al.*, 1986). For this, 4 ml Percoll solution (60%) was pipetted into a 15 ml Falcon tube and 10 ml of asexual *Plasmodium* culture were carefully layered onto the Percoll solution without mixing parasite culture and Percoll. Layered parasite culture was centrifuged at 2000 rpm for 8 min. After centrifugation, a dark ring was visible between a medium layer and a Percoll layer containing schizont stage parasites. At the bottom of the Falcon tube a pellet containing the ring stage parasites was visible. The medium was carefully removed and the parasite layer including the Percoll layer was transferred into a fresh 15 ml Falcon. Parasites were washed in 10 ml fresh RPMI medium and after washing, the schizont pellet was pipetted into an Eppendorf tube containing 300 µl fresh human blood and 500 µl RPMI complete medium. The Eppendorf tube was placed on a thermo shaker and parasite culture was incubated for 30 min at 37°C, 600 rpm. After shaking, parasites were re-cultivated in a Petri dish and incubated at 37°C.

3.3.6   Transfection of *P. falciparum* parasites via electroporation

The correct plasmids were transfected in *P. falciparum* using electroporation. In general, two different methods were used to transfect the *Plasmodium* parasites. For the transfection of ring stage parasites (Fidock & Wellems, 1997), 100 µg DNA were precipitated per transfection and resuspended in TE buffer and cytomix (see 3.1.8). The asexual parasite culture was grown to a high parasitemia between 5 – 10%, and the culture was synchronized via D-sorbitol to deplete later developmental stages. The synchronized culture was centrifuged at 2000 rpm for 5 min and supernatant was discarded. The pellet containing the ring stage parasites was resuspended in prepared DNA-cytomix solution, transferred into an electroporation cuvette (2 mm, Bio-Rad). For electroporation, the Gene Pulser Xcell (program: 310 V, 950 µF, ∞ Ω) was used. After electroporation, the parasite culture was transferred into a fresh Petri dish and supplied with RPMI complete medium. The selection drug was added 24 hours after transfection of the parasites and medium was changed daily for the first week, and afterwards every second day.

For transfection of late schizont stages (Moon *et al.*, 2013), parasite culture was synchronized using 60% Percoll two days prior to the transfection (see 3.3.5). The DNA (50 µg in TE buffer and Amaxa transfection buffer) was precipitated as described in 3.1.8. On

the day of transfection, the synchronous culture was synchronized again using 60% Percoll and the isolated schizont pellet was mixed with the prepared DNA mix, and transferred into an electroporation cuvette (2 mm, Bio-Rad). For the electroporation, the Amaxa system (Nucleofector II AAD-1001N, program: U-033) was used. After electroporation, the parasite mix was pipetted into a 1.5 ml Eppendorf tube containing 500 µl fresh RPMI complete medium and 300 µl fresh human blood. The Eppendorf tube was incubated on a thermo shaker for 30 min at 37°C and 600 rpm to support invasion of merozoites into new red blood cells. After shaking, the parasite culture was pipetted into a Petri dish and selection drug was added 24 hours later. For one week, medium was changed daily and afterwards every second day.

3.3.7 Selection of transgenic parasites via selection linked integration (SLI)

Transgenic parasites were selected by using SLI (Birnbaum *et al.*, 2017). For this, transgenic asexual *Plasmodium* cultures were selected with WR99210 for the presence of episomal construct and were grown to a parasitemia of 4 – 6%. The selection marker G418 was added to a final concentration of 400 µg/ml. In case of yDHODH resistance, 0.9 µM DSM1 were used for selection process. For the first seven days, RPMI complete medium containing the respective drug was changed daily and parasitemia was checked the latest on day three for surviving parasites. After the first week, medium was changed every second day and the culture was checked regularly every week for growing parasites until transgenic cultures came up again. In case of reappearing parasites, parasite culture was harvested, gDNA was isolated via the QIAamp DNA Mini Kit (see 3.1.9), and integration check was performed by PCR to confirm the correct integration of the construct in the genomic locus of the parasites (see 3.1.10). If residue of original locus were still traceable in the transgenic parasite culture, selective pressure, using either G418 or DSM1, was continued for another two weeks, after which the integration check was repeated. In general, the selection process was performed for up to three months and checked continuously for reemerging parasites. In case there were no reappearing parasites detected, cultures were discarded after this time.

3.3.8 Saponin lysis of *P. falciparum* infected erythrocytes

The detergent saponin is used to selectively permeabilize the membrane of the red blood cell, as well as the PVM. Due to its special lipid composition, the PPM around the parasite stays intact (Benting *et al.*, 1994; Ansorge *et al.*, 1996). Saponin lysis was performed to release the *Plasmodium* parasite from the RBC and deplete hemoglobin from parasite extract. For this, 10 ml parasite culture containing a high parasitemia of the desired developmental stage were harvested and centrifuged at 4000 g for 5 min. After this, the supernatant was removed and the pellet was washed once in 10 ml 1xPBS (room temperature, RT) and centrifuged again. The supernatant was discarded, and parasite pellet was resuspended in 5 ml ice-cold 0.03% saponin solution and incubated on ice for 10 min. Next, the parasite culture was pelleted at 4000 g for 15 min and the pellet was transferred to a 2 ml Eppendorf tube and centrifuged at maximum speed for 3 min. The pellet was washed with ice-cold 1xPBS until supernatant was clear and free of hemoglobin. Meanwhile, a master mix containing 450 µl lysis buffer and 50 µl PIC inhibitor was prepared. After washing the pellet, it was resuspended in the lysis buffer / PIC mix (volume dependent on the size of the pellet) and 1 – 2 µ PMSF was added and incubated on ice. The parasite suspension was centrifuged, and supernatant (in case of soluble proteins) was transferred into a fresh 1.5 ml Eppendorf tube. The supernatant was mixed with 30 µl 4x SDS + 1 M DTT and samples were heated at 85°C for 5 min. In case of the *Pf*Kelch13 proteins, the saponin samples were not heated at 85°C for 5 min but treated with pre-warmed 4x SDS + 1 M DTT solution to avoid depletion of the protein. For SDS-PAGE, 10 µl sample and 8 µl marker were loaded onto the gel. In general, saponin samples could be stored at -20°C after heating to 85°C, but for *Pf*Kelch13 SDS-PAGE must be performed immediately after saponin pellet preparation to avoid depletion of the protein.

3.3.9 Inactivation of parasite proteins by knock sideways system

For inactivation of a target protein, the knock sideways system was used (Haruki *et al.*, 2008; Robinson *et al.*, 2010; Xu *et al.*, 2010). An asexual parasite culture was split into two parts, 2 ml culture were kept as control and 2 ml culture were treated with 250 nM rapalog (A/C heterodimerizer AP21967, Clontech). After 1, 2, 4, and 22 hours a Giemsa smear of the cultures was prepared (see 3.3.2) and assessed by light microscopy and additionally slides were prepared for fluorescence microscopy to estimate target mislocalization via live cell imaging.

3.3.10 Flow cytometry-based growth assay

To estimate the growth of a cell line after inactivation of a target protein, a flow cytometry-based growth assay was performed over a period of 5 days. To start the growth assay, the parasitemia of an asexual parasite culture was measured by flow cytometry (Malleret *et al.*, 2011). For this, the parasite cells (20 µl) were stained for 20 min at room temperature with a master mix containing 80 µl RPMI complete medium, 1 µl Hoechst33342 (final concentration: 4.5 µl/ml) working solution and 1 µl DHE (0.5 µg/ml) working solution. After 20 min, the staining was stopped by adding 400 µl of 0.003% glutaraldehyde in RPMI complete medium. Measurement was performed using the LSR flow cytometer and the FACSDiva (BD). In total, 100 000 events were counted to estimate the parasitemia of the culture. After measuring the parasitemia, the parasite culture was adjusted to 0.1% parasitemia and split into two 2 ml Petri dishes. One dish was kept untreated as control, while to the other dish 250 nM rapalog (A/C heterodimerizer AP21967, Clontech) was added. Parasites were re-cultivated at 37°C and every 24 hours, parasitemia was measured by flow cytometry for 4 further days (5 days in total) to assess the impact of the mislocalization of a target protein on parasite growth. Medium and rapalog were changed daily, and additional Giemsa smears were taken each day.

3.3.11 *In vitro* ring-stage survival assay[0-3h] (RSA)

The standard ring-stage survival assay was performed to test whether parasites are resistant to ART. RSAs were performed as previously described (Witkowski & Amaratunga *et al.*, 2013). Percoll gradient (60%) was used to isolate mature schizonts from an asynchronous parasite culture containing 4 – 6% schizonts (see 3.3.5). The mature schizonts were allowed to invade fresh red blood cells (2 ml volume; 2.5% hematocrit per sample, meaning a hematocrit of 5% for control and DHA fraction in total) at 37°C for three hours in a 6 well plate and were then synchronized again in a 2 ml Eppendorf tube using 1 ml 5% D-sorbitol per tube to obtain ring stage parasites at the age of 0 – 3 hours (see 3.3.5). The rings were washed in a 2 ml Eppendorf tube twice with 1 ml fresh RPMI complete medium (3700 g; 1.5 min) and split in two fractions (2 ml volume per fraction in a 6 well plate; 2.5% hematocrit per sample). One part was treated with 700 nM DHA for 6 hours, whereas the other fraction was left untreated as control. For a standard RSA, after the 6 hours drug pulse, DHA was removed by washing the samples three times, each with 1 ml fresh RPMI complete in a 2 ml Eppendorf tube. After washing, the parasites were re-

cultivated for another 66 hours in a 6-well plate (2 ml volume) and Giemsa smears were prepared after 72 hours to determine the proportion of parasites that survived the DHA treatment (see 3.3.2). For control, at least 80 parasites and for DHA-treated fraction at least 150 panels were counted. To estimate parasite survival, parasitemia of the control was compared to the parasitemia of the DHA treated fraction. Parasites were defined as resistant, when mean survival rate exceeded the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013).

To assess the effect of inactivation of a special target protein on the survival rate, the standard RSA was slightly modified. For this, parasites were cultured in the presence (inactivation via knock sideways system) or absence (control) of 250 nM rapalog (A/C heterodimerizer AP21967, Clontech). The start of knock sideways was induced depending on the respective target protein. For *Pf*UBP1 and *Pf*KIC7, inactivation was initiated 1 hour, for *Pf*AP-2µ and *Pf*2102 7 hours, for 3D7, *Pf*Kelch13 and *Pf*MyosinF 12 hours, for *Pf*Eps15 18 hours, and additionally for *Pf*MyosinF 24 hours prior to Percoll purification of schizonts. The pre-incubation of 3D7 parasites served as a control to check for potential effects of rapalog alone. Rapalog exposure was maintained either until 1 hour after addition of DHA (*Pf*UBP1/*Pf*KIC7/*Pf*Eps15/*Pf*AP-2µ/*Pf*2102), or up to the removal of DHA in the parasites for which no growth defect was expected (3D7/*Pf*Kelch13/*Pf*MyosinF). When rapalog was removed prior to DHA removal, parasite cultures were washed three times with 1 ml fresh RPMI complete medium (using a 2 ml Eppendorf tube) and 700 nM DHA were added again until 6 hours of drug exposure was completed. After the 6 h DHA pulse, DHA (and rapalog, where still present) was removed by washing the cultures for three times in 1 ml RPMI complete medium (using a 2 ml Eppendorf tube). Afterwards, the cultures were grown for another 66 hours in a 6-well plate (2 ml volume, 2.5% hematocrit) to complete the RSA. At the end (72 hours), Giemsa smears and parasite survival rate was calculated.

3.3.12 Consecutive *in vitro* ring-stage survival assay[0-3h] (RSA)

A consecutive RSA was performed to check whether already ART resistant parasites can become even more resistant by challenging them in series. For the consecutive RSA, a standard *in vitro* RSA was performed as described before (see 3.3.11) (Witkowski & Amaratunga *et al.*, 2013). In general, three technical and two biological replicates were performed regularly. At 72 hours, Giemsa smears were taken and the resistant parasites which survived the DHA treatment (DHA treated sample) were re-cultivated in a new Petri dish and grown at 37°C. After several days of recovery, these resistant parasites were

tested again in a new round of *in vitro* RSA. This procedure was continuously repeated for 30 weeks in a row, and parasite survival rate was determined by Giemsa smear.

3.3.13 Bloated food vacuole assay

The bloated food vacuole assay was used to check whether hemoglobin uptake is impaired by the inactivation of a specific target protein. Here, a bloated food vacuole phenotype appears when hemoglobin uptake is impaired. For this, an asynchronous parasite culture was synchronized twice using 5% D-sorbitol with 10 hours treatment apart to obtain ring stage parasites of 10 – 18 hours age. The rings were re-incubated at 37°C for another 8 hours to grow parasites into trophozoites of 18 – 26 hours. A sample was removed for imaging to estimate the size of the food vacuole at time point 0 and the parasite culture was split into two 1 ml cultures, and both were treated with 33 µM E64 protease inhibitor (Sigma Aldrich). To one culture rapalog was added to a final concentration of 250 nM, whereas the other cell culture was kept as control. The parasite cultures were incubated at 37°C for 8 hours and then microscopy samples were removed and stained at room temperature with 4.5 µg/ml dihydroethidium for 20 min. After staining, the samples were washed once with fresh RPMI complete medium and immediately imaged. The size of the bloated food vacuole was measured by DIC image using ImageJ (30 cells were imaged per approach) and number of vesicles was counted by DIC images. Before imaging, the operators were blinded to the conditions of the sample.

3.3.14 Cytochalasin D inhibitor treatment of *P. falciparum*

Cytochalasin D is an inhibitor that destabilizes actin filaments. To check whether inhibitor treatment influences parasite development, an asynchronous parasite culture was separated into two parts and cultivated for 1 hour either in the presence of 10 µM Cytochalasin D (Calbiochem), or absence of inhibitor (control) at 37°C. After treatment, cells were imaged immediately, and relative position of *Pf*MyosinF foci to *Pf*Kelch13 in trophozoite stages was assessed. For this, *Pf*MyosinF and *Pf*Kelch13 foci per cell were counted and classified into 3 conditions. Co-localization of *Pf*MyosinF and *Pf*Kelch13 was defined as total overlap, when yellow color was detected in the merge, partial co-localization was defined as 50% overlap, when yellow signal appeared in the merging region, and non-co-localization events as defined as touching foci that did not overlap or were further apart.

3.3.15 Parasite fitness assay

The fitness assay was used to compare the growth of different cell lines over several weeks (Tirrell *et al.*, 2019). For this, the cell lines were synchronized with 60% Percoll (see 3.3.5) and allowed to re-invade fresh RBCs for 6 hours at 37°C. After 6 hours, the parasites were synchronized again using 5% D-sorbitol to obtain a culture of 0 – 6 hours rings. Parasites were cultured at 37°C for another 20 – 24 hours and parasitemia was measured via flow cytometry (staining see 3.3.10) (Birnbaum *et al.*, 2017). For starting the fitness assay, the different cell lines were co-cultivated in a 5 ml Petri dish in a 1:1 ratio with 3D7 control based on the parasitemia measured by flow cytometry. The co-cultivated cell lines were grown either in RPMI complete or low amino acid medium. Cells were stained with Hoechst33342 (1:5000) for 10 min and the proportion of GFP-positive parasites was assessed by fluorescence microscopy every two days until one parasite line was overgrown by the other cell line of the co-culture (ratio of 95:5). Per condition (RPMI complete or Low AA), the non-linear regression curves were derived from the respective raw curves, expressing the proportional amount of GFP positive parasites per total parasitemia. The fitness cost per generation of the different strains were calculated based on the indicated functions (raw curves and calculations in Appendix H.1 – H.4). For this, in a first step the trendline factor from the indicated functions was duplicated (assumption that one generation corresponds to two days), and growth factor per generation was differentiated. Fitness cost per generation were calculated by subtracting the differentiated value from 1 (1 representing 100% fitness).

3.3.16 Measurement of protein amount by fluorescence intensity

Measurement of protein amount via fluorescence intensity was performed as described before (Birnbaum *et al.*, 2020). To determine the protein amount of different cell lines, parasites were synchronized twice using 5% D–sorbitol (see 3.3.5) in intervals of two days, to obtain 0-18 hours ring stage parasites. After the second sorbitol synchronization, the cell lines were cultivated at 37°C for another 2 hours, then the GFP signal of the ring-stage parasites was detected by fluorescence microscopy. Intensity of the GFP signal in comparison to intensity of the background signal was measured via ImageJ and correlated with the respective area of the GFP signal to obtain the total fluorescence. The mean fluorescence intensity was calculated via the average from three independent experiments.

3.4 Biochemical methods

3.4.1   Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight (Laemmli, 1970). To be able to separate the proteins based on their molecular weight, SDS was used to denaturate the proteins by unfolding them and providing a negative charge irrespective of their original charge. The SDS sample buffer was supplied with DTT to reduce the disulfide bonds of the protein and to promote denaturation. For the separation of the polypeptides, polyacrylamide gels of 6%, 8%, and 10% concentration were prepared (see 2.9.3). First, a casting gel stand (Bio-Rad) was assembled and tested on its impermeableness using dH_2O. Next, the separation gels were cast between two glasses in the casting gel stand and polymerized for 30 min. To plane the gel border, isopropanol was carefully layered onto the gel during polymerization. When the gel was totally polymerized, isopropanol was removed and stacking gels were prepared (see 2.9.3) and carefully cast onto the polymerized separation gels. Directly after casting the stacking gel, a comb was put into the gel and the stacking gel was polymerized for another 30 min. When polymerization was completed, the gels were placed in an electrophoresis chamber (Bio-Rad) which was filled with 1x running buffer and gel combs were removed. The prepared saponin pellets diluted in 4x SDS buffer + 1 M DTT were loaded into the gel slots. In general, 8 µl pre-stained protein ladder (Thermo Fisher) and 10 µl protein samples were loaded into the slots. The SDS-PAGE was performed for the first 15 min at 100 V to carefully run the samples into the stacking gel, and then electrophoresis was continued at 150 V in the separation gel. SDS-PAGE was stopped when the dye front of the marker reached the bottom of the gel.

3.4.2   Western Blot analysis

Proteins were separated according to their molecular weight by SDS-PAGE (see 3.4.1). After the electrophoresis, proteins were transferred to a nitrocellulose membrane to identify them with specific antibodies detecting the immobilized proteins (Towbin *et al.*, 1979). For this, the blotting cassette was assembled. Prior to layering, all components were soaked in 1x transfer buffer, and the layering was started with the blotting foam, followed by three blotting papers. The SDS gel was carefully removed from the glass plate and layered on

the blotting paper. All air bubbles were removed, and blotting set up was constantly wetted to avoid destruction of the proteins. The gel was covered with a nitrocellulose membrane (PROTRAN), followed by three blotting papers and a second blotting foam. The blotting cassette was closed, placed in the blotting chamber, and filled up with ice-cold 1x transfer buffer. Icepacks were added to the chamber and blotting was performed at 299 mA, 390 V for 1.5 h at 4°C. When the blotting was completed, the blotting cassette was disassembled, and the nitrocellulose membrane containing the transferred proteins was blocked for 1 hour in 5% milk in 1x PBS at room temperature to prevent unspecific binding of antibodies.

3.4.3   Immunodetection of proteins

To specifically detect the proteins blotted on the nitrocellulose membrane after blocking, the membrane was incubated with the primary antibody (see 2.1.1 in 2% milk in 1xPBS) overnight at 4°C. Next day, the primary antibody was removed by washing the membrane two times in 1x PBS-Tween for 15 min. After washing, the secondary antibody conjugated to horse radish peroxidase (HRP) (see 2.1.2 in 2% milk in 1x PBS) was added to the membrane and incubated for 1 hour at room temperature. After this incubation, the membrane was washed three times in 1x PBS-Tween, once in 1x PBS, and once in dH_2O. For immunodetection, the membrane was placed on transparent film, and the ECL-Western blot detection Kit (Bio-Rad) was used to detect HRP by chemiluminescence. After 1 min incubation with the detection solutions, the signal intensities of the proteins were detected by ChemiDoc XRS+ imaging system (Bio-Rad). In total, the exposure time was 2 – 2400 sec wherein 35 pictures were taken. For loading control, the membrane was washed three times in 1x PBS after signal detection and blocked for 1 hour in 5% milk in 1x PBS. Antibody staining and imaging was performed as described before.

3.5 Microscopy

3.5.1   Live cell imaging

Live cell imaging was performed using the Zeiss AxioImager M1 equipped with a Hamamatsu Orca C4742-95 camera and the Zeiss Axiovision software (v 4.7). Here, a 100x / 1.4 – numerical aperture lens, and a 63x / 1.4 – numerical aperture lens was used. The microscopy images were processed via Corel PHOTO-PAINT X8 (64-bit) and CorelDRAW

X8 (64-bit). For staining of parasite nuclei, 500 µl parasite culture was stained either with DAPI (final concentration 1 µg/ml), or Hoechst33342 (1:5000) for 10 min at room temperature. After staining, parasite culture was washed once in fresh RPMI complete medium, centrifuged at 3700 rpm for 1 min and 4 µl parasite pellet was dropped onto a glass slide and covered with a coverslip.

3.5.2   Confocal imaging of parasites following DHA pulse in standard *in vitro* RSA

To follow up the development of ART resistant parasites after a DHA pulse, RSA was combined with confocal imaging. For this, a standard *in vitro* RSA was performed with K13^{C580Y} parasites as described in 3.3.11, and immediately after removal of DHA after 6 hours treatment parasites were prepared for imaging by confocal microscopy. The microscopy was performed using the Olympus FluoView 1000 confocal microscope. To detect the parasites, image stacks (30 Z-layers) were taken with the 488 nm laser line, using a 60x (1.35 numerical aperture) plan apochromate oil immersion lens and a 2.0-fold zoom via the FluoView software. Images were taken at 3 different time points: directly after DHA removal (0 h), 24 hours after DHA removal (24 h), and 39 hours after DHA removal (39 h). For image processing, the Imaris x64 7.8 (Bitplane) software and Corel Suite X8 were used. Snapshots from the DIC images were prepared and area of parasite cell was measured after 24 hours via ImageJ to assess the mean size of control and ART treated fraction. The 39 h time point was used to estimate the respective condition of the parasite and classify them into reinvading parasites, trophozoite or schizont stages or dead parasites.

3.5.3   Immunofluorescence assay (IFA) performed with acetone fixed cells

To detect endogenously expressed parasite proteins, IFAs with acetone fixed cells were performed. For this, 500 µl of parasite culture containing ~5% parasitemia of all developmental stages were harvested and pelleted at 2000 rpm for 1 min. Parasite cells were washed twice with 1x PBS and were resuspended in a suitable volume of 1x PBS to obtain a hematocrit of ~ 3%. Of this parasite suspension, 50 µl were taken and transferred to a 10 well IFA slide aspirating the parasite suspension repeatedly per well to leave only a thin cell monolayer behind. The cells on the IFA slide were air-dried and fixed in fresh 100% acetone for 30 min. After fixation, IFA slides were air-dried and then rehydrated with 1x PBS. Each well was washed twice with 1x PBS and slide was placed in a humid chamber.

For epitope detection, antibody solution was prepared by diluting the primary antibody (see 2.1.1) in 3% BSA in 1x PBS supplied with 100 µg/ml ampicillin. The parasite cells were incubated with the primary antibody solution in the humid chamber for 1 hour at room temperature. After incubation, the IFA wells were washed five times with 1x PBS. The secondary antibody solution (see 2.1.2) was prepared by diluting the respective antibody in 3% BSA in 1x PBS. Parasite cells were incubated with the secondary antibody and 1 µg/ml DAPI staining solution for 1 hour at room temperature in a humid chamber. After incubation, IFA wells were washed five times with 1x PBS and slides were dried at 37°C in the incubator. Small drops of 1x PBS buffered glycerol were pipetted between the wells, a coverslip was placed onto the slide and sealed with nail polish. Acetone-fixed cells were imaged using the Zeiss Axioscope M1 as described before (see 3.5.1). For storage, the IFA slides were wrapped in aluminum foil and kept at 4°C.

3.5.4 Immunofluorescence assay (IFA) performed with formaldehyde / glutaraldehyde-fixed cells

For better preservation of cell morphology and detection of the endogenously expressed parasite proteins, a formaldehyde/glutaraldehyde-fixed immunofluorescence assay was performed. At the beginning, 1 ml of parasite culture was harvested from an asexual cell culture growing at a parasitemia of ~4%. The parasite culture was centrifuged at 1800 rpm for 5 min and pellet was washed twice in 1x PBS. After washing, the pellet was resuspended in 750 µl fixing solution (freshly prepared, see 2.9.4) and incubated at room temperature for 30 min. Next, the parasite suspension was centrifuged at maximum speed for two minutes and cell were permeabilized with 0.5% Triton X-100 in 1x PBS for 10 min at room temperature. Afterwards, the culture was pelleted and washed three times with 1x PBS. After washing, the cells were spun down at 16 000 rpm for 2 min and unspecific antigen binding sites were blocked for one hour at room temperature with 100 µl 3% BSA in 1xPBS with ampicillin (100 µg/ml). After blocking, cells were pelleted, washed once in 1x PBS, and incubated rolling with 100 µl of primary antibody solution (see 2.1.1 in 3% BSA in 1x PBS) for one hour at room temperature (alternatively at 4°C overnight). Next, the cells were pelleted again, washed three times in 1x PBS, and spun down at 3000 rpm for 2 minutes. The secondary antibody solution was prepared by diluting the respective antibody (see 2.1.2) in 3% BSA in 1x PBS with ampicillin (100 µg/ml), and additionally 1 µg/ml DAPI staining solution was added. The cells were resuspended in 100 µl secondary antibody solution, protected from light, and incubated rolling for one hour at room temperature.

Secondary antibody solution was removed by pelleting the cells and washing the parasites three times in 1x PBS. After washing, the parasite cells were spun down at 3000 rpm for 2 min and prepared for imaging. The cells were imaged using the Zeiss Axioscope M1 as described before (see 3.5.1). For storage, 100 µl 1xPBS with 100 µg/ml ampicillin were added to the pellet. To prevent bleaching, aluminum foil was wrapped around the tube and cells were kept at 4°C.

4 Results

4.1 Functional characterization of *Pf*MCA2 (PF3D7_1438400)

4.1.1 Localization of *Pf*MCA2 (PF3D7_1438400)

Analysis of the Kelch13 compartment using DiQ-BioID and quantitative mass spectrometry revealed several candidates of *Pf*Kelch13 interactors (Birnbaum *et al.*, 2020). One of the hits that so far was not validated to be present at the *Pf*Kelch13-defined compartment was the protein *Pf*MCA2 (PF3D7_1438400). Since only little information about this protein exists, experiments were performed to first determine whether it indeed is a Kelch13 compartment protein and to further characterize it. Previously, several attempts employing SLI to C-terminally tag the protein with a sequence coding for FKBP domains and GFP failed (Birnbaum J., personal communication). Therefore, it was here attempted to tag *mca2* with the sequence encoding 3xHA by modification of the original genomic locus using SLI, resulting in the cell line MCA2^{wt}-3xHA. Correct integration of the SLI construct was confirmed by PCR (Fig 11 A) and expression of the HA-tagged *Pf*MCA2 was verified by western blot analysis (Fig 11 B).

To investigate the localization of *Pf*MCA2 in the parasite, anti-HA IFAs were carried out with formaldehyde/glutaraldehyde-fixed cells. Fluorescence microscopy with these parasites showed that *Pf*MCA2 is expressed throughout the asexual cycle of *P. falciparum* (Fig 11 C). In ring stage parasites, one focus was visible whereas in trophozoite stage parasites two or more foci were detected. Interestingly, here one focus was often located close to the FV (yellow arrows Fig. 11 C) while the other focus was observed at structures visible in DIC that may represent vesicles at cell periphery (white arrows Fig. 11 C). In schizonts, detection of *Pf*MCA2 was more difficult since the signals was more diffuse at this stage but also in schizonts *Pf*MCA2 was seen in one focus localizing close to the FV.
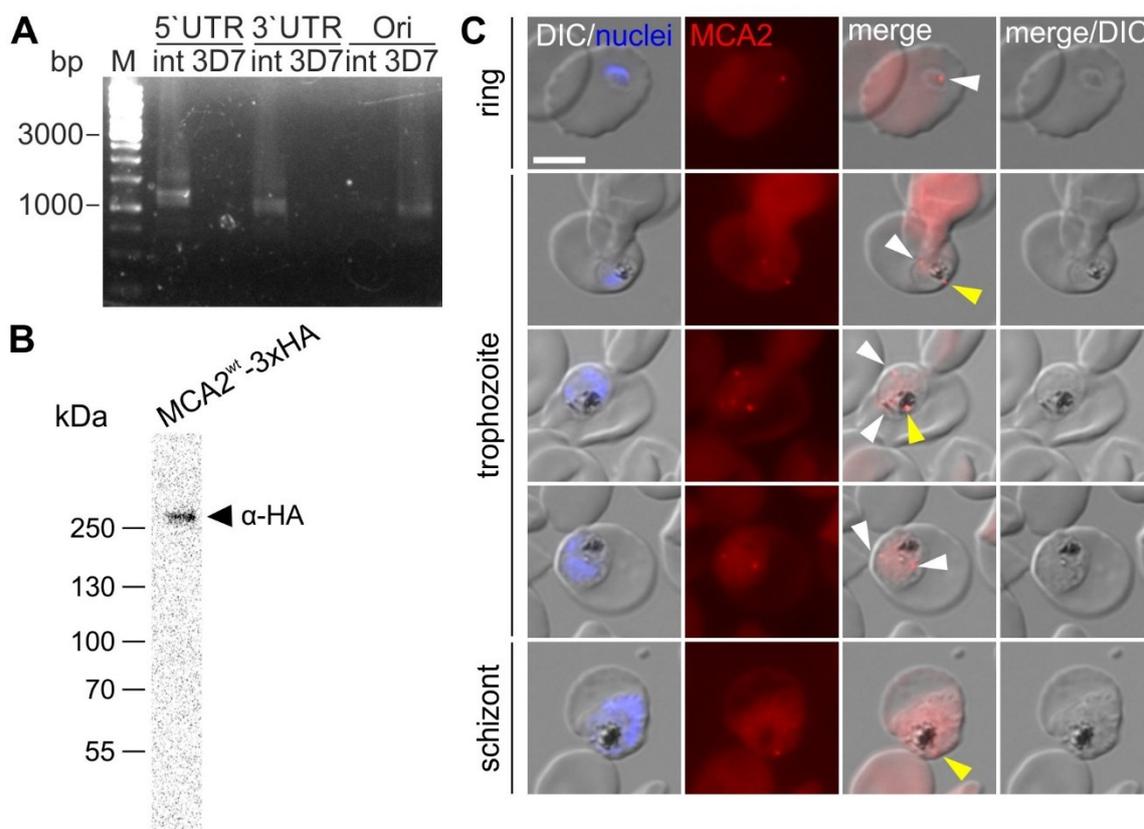
Figure 11) *Pf*MCA2 (PF3D7_1438400) localizes close to food vacuole and vesicles. **A)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain MCA2wt-3xHA parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 1273 bp) and 3`-integration (3`UTR, 1153 bp) and to demonstrate absence of original locus in the MCA2wt-3xHA parasites (Ori, 1100 bp). 3D7 gDNA was used as reference (ori 3D7 1100 bp). **B)** Western blot analysis with protein extracts of saponin-lysed parasites of MCA2wt-3xHA parasites. α-HA (from rat) was used to detect MCA2wt-3xHA (calculated molecular weight (MW) of 281 kDa). Molecular weight standard is indicated in kDa. Full blots are shown in Appendix F.2. **C)** IFA images of formaldehyde/glutaraldehyde-fixed MyoFwt-3xHA parasites. α-HA (from rat) was used to detect MCA2wt-3xHA. Nuclei were stained with DAPI. Yellow arrow, marks foci detected at the FV; white arrow, marks foci detected at vesicles at cell periphery; *bp*, base pairs; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *int*, integrant; *ori*; original locus; *wt*, wildtype; *HA*, hemagglutinin; *DIC*, differential interference contrast. scale bar, 5 µm.

4.1.2   *Pf*MCA2 is part of the Kelch13 compartment

For verification of the presence of *Pf*MCA2 at the Kelch13 compartment, the cell line MCA2wt-3xHA was transfected with an episomal plasmid containing a sequence coding for *Pf*Kelch13 N-terminally tagged with mCherry, resulting in the cell line MCA2wt-3xHA+K13 (Fig 12 A).

To analyze the relative position of *Pf*MCA2 and *Pf*Kelch13 in the parasite cell, MCA2^wt^-3xHA+K13 parasites were acetone fixed and imaged using fluorescence microscopy (Fig 12 B). This showed similar to the formaldehyde/glutaraldehyde fixed parasites (see section 4.1.1), *Pf*MCA2 was detected throughout the asexual cycle of *P. falciparum* and one or more foci were found in close proximity to the FV. Episomal expressed *Pf*Kelch13 was also found in all asexual stages and was detected close to *Pf*MCA2. Quantification of the relative localization of *Pf*MCA2 and *Pf*Kelch13 was performed (Fig 12 C). Here, *Pf*MCA2 and *Pf*Kelch13 were defined as co-localizing when the foci totally overlapped (yellow color in merge). *Pf*MCA2 and *Pf*Kelch13 were scored as partially co-localizing when around half of the foci size overlapped (yellow signal in the merging region), whereas both proteins were defined as non-colocalizing when they were closely located but the foci did not overlap. This revealed that 44% of *Pf*MCA2 foci co-localized with *Pf*Kelch13. Partial overlap was calculated for 24% of *Pf*MCA2 foci the cases, whereas 32% *Pf*MCA2 foci did not co-localize with *Pf*Kelch13. This data indicates that *Pf*MCA2 is part of the Kelch13 compartment but is also found at other foci.
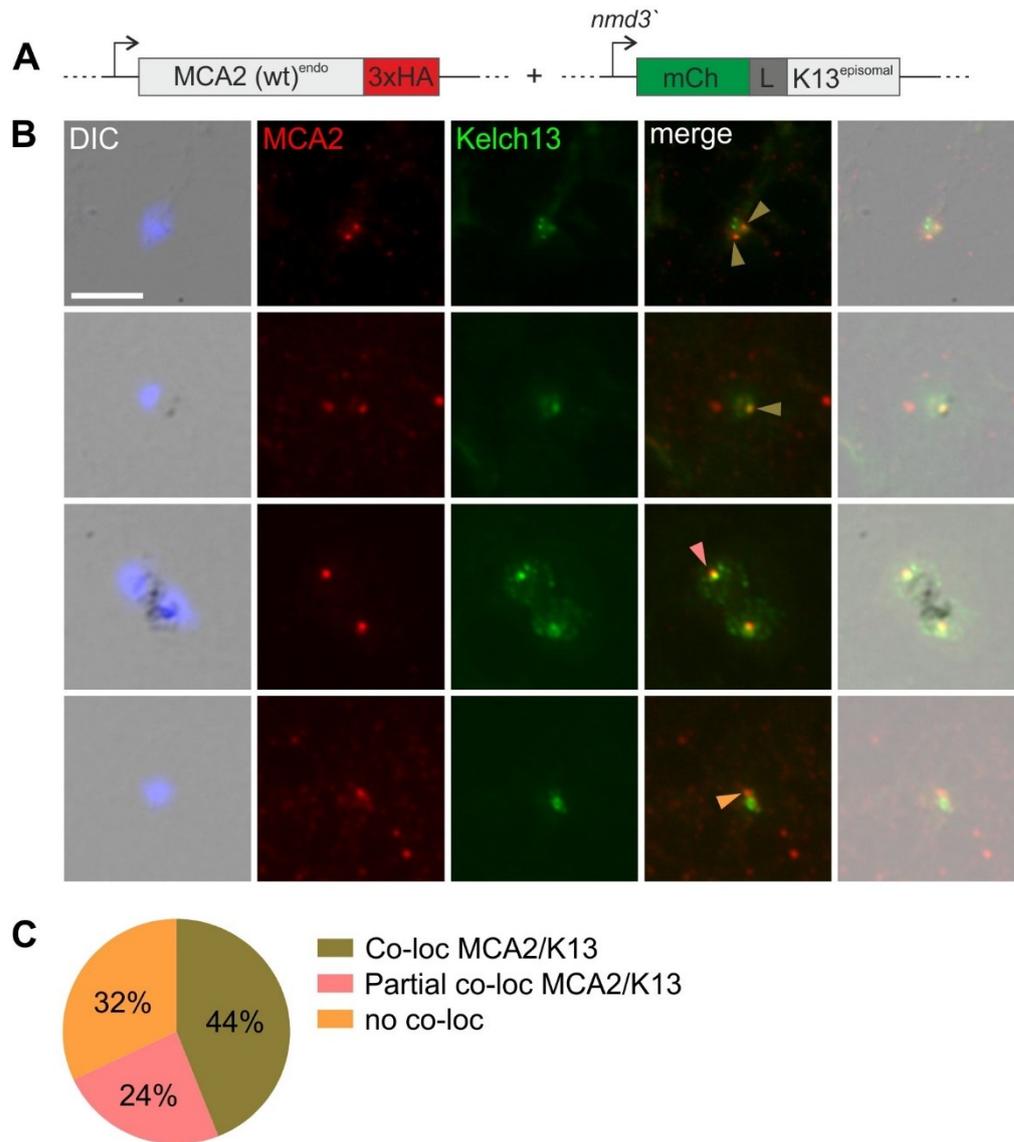
Figure 12) Relative localization of *Pf*MCA2 to *Pf*Kelch13. **A)** Schematic of modified endogenous locus of MCA2wt-3xHA parasites transfected with episomal mCherry-K13 plasmid. **B)** IFA images of acetone-fixed MCA2wt-3xHA+K13 parasites. α-HA (from rabbit) was used to detect MCA2wt-3xHA. α-RFP (from rat) was used to detect mCh-Kelch13. Nuclei were stained with DAPI. **C)** Quantification of spatial arrangement of *Pf*MCA2 and *Pf*Kelch13 foci. Shown is percentage of *Pf*MCA2 foci that co-localize (full overlap when all of the *Pf*MCA2 focus resulted in a yellow signal in the merge), partially co-localize (partial overlap of foci resulting in a yellow signal in the merging region) or did not co-localize (foci closely located but no overlap). One acetone IFA was performed, and n = 30 cells were scored. *wt*, wild type; *HA*, hemagglutinin; *mCh,* mCherry; *L,* linker; *DIC*, differential interference contrast; *green arrow* marks co-localizing foci, *pink arrow* marks partially co-localizing foci; *orange arrow* marks no co-localization between *Pf*MCA2 and *Pf*Kelch13; scale bar, 5µm.

4.1.3 SNP found mainly in African field isolated leads to truncation of *Pf*MCA2 and renders parasite resistant to ART

By screening of the MalariaGEN *Plasmodium falciparum* Community Project (MalariaGEN *et al.*, 2021) a SNP at amino acid position 1344 of *Pf*MCA2 was found leading to a conversion of the corresponding codon into a stop (Fig 13 A). According to MalariaGEN, this SNP is found in Africa with a mean prevalence of 52% and in South Asia with 5% prevalence. Previous data showed that truncation of *Pf*MCA2 is possible for parasite growth and renders parasite resistant to ART (Birnbaum *et al.*, 2020). To investigate whether Y1344Stop also induces ART resistance, *Pf*MCA2 was disrupted at the respective position using SLI, leading to a cell line expressing a truncated version of *Pf*MCA2 (MCA2^{Y1344Stop}-GFP). After selection of integrants, correct integration of the construct was confirmed via PCR (Fig 13 B) and correct expression of the truncated protein was verified by western blot analysis (Fig 13 C). Live cell imaging of MCA2^{Y1344Stop}-GFP parasites revealed that the truncated version of *Pf*MCA2 is expressed throughout the asexual replication in the infected red blood cell similar to wild type *Pf*MCA2 (Fig. 13 D). In agreement with the findings with the MCA2^{wt}-3xHA parasites (see Fig 11 C), ring stage parasites showed a single focus of MCA2^{Y1344Stop}-GFP per cell, while in trophozoite stages two or more foci were detectable (Fig. 13 D). In most of the cells one focus was localized next to the FV, whereas the other foci are present in cell periphery often close to vesicular structures (Fig. 13 D, orange, and white arrows, respectively).

Performing standard RSA with the MCA2^{Y1344Stop}-GFP cell line showed that truncation of the native *Pf*MCA2 locus at amino acid position 1344 rendered parasite resistant to ART with a mean parasite survival rate of 1,64% that is higher than the defined cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013) (Fig 13 E). This is in line with previous results demonstrating that disruption of *Pf*MCA2 at amino acid position 57 confers tolerance to ART (Birnbaum *et al.*, 2020).

In summary, the data indicates that disruption of *Pf*MCA2 at amino acid position 1344 is dispensable for parasite development and does not impair localization of *Pf*MCA2. While this might indicate that this SNP found mainly in Africa may render parasite resistant to ART, the analysis of this sequence region from African parasite isolates revealed that it always seems to occur with a SNP at gene position 4030 which leads to an amino acid change from Y to K, rather than a stop (RNA sequencing data kindly provided by Anna Bachmann and Thorsten Thye). Hence, this SNP is likely not relevant for ART resistance in

the field, but the truncation analyzed here confirms the importance of *Pf*MCA2 for ART resistance.
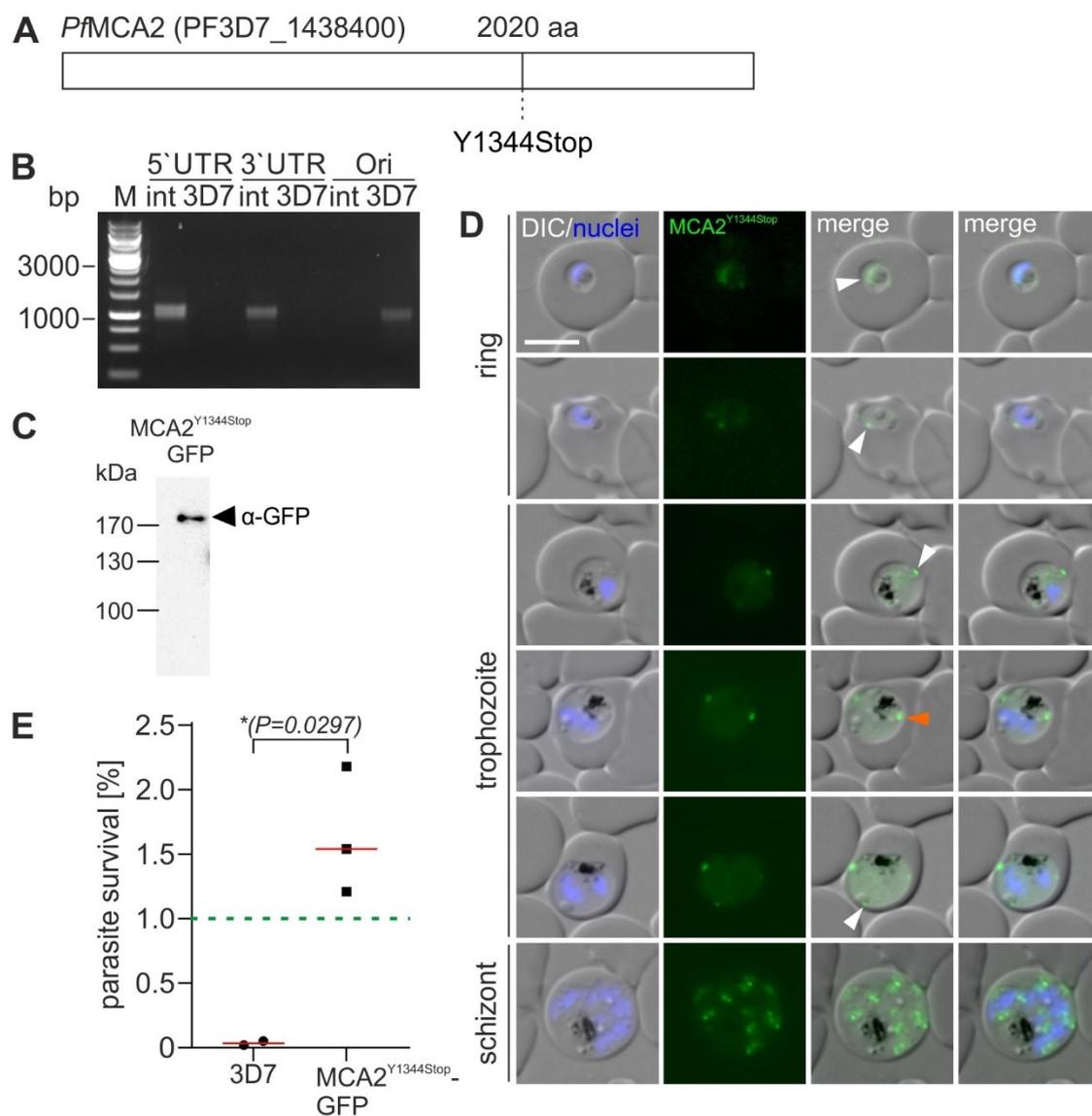
Figure 13) SNP (Y1344Stop) found in field isolates that when introduced into 3D7 leads to truncation of *Pf*MCA2 and renders parasite resistant to ART. **A)** Scheme showing position of SNP in MCA2[Y1344Stop]-GFP cell line. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain MCA2[Y1344Stop]-GFP parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 1188 bp) and 3`-integration (3`UTR, 1120 bp) and to demonstrate absence of original locus in the MCA2[Y1344Stop]-GFP parasites (Ori, 1070 bp). 3D7 gDNA was used as reference (ori 3D7 1070 bp). **C)** Western blot analysis with protein extracts of saponin-lysed parasites of MCA2[Y1344Stop]-GFP parasites. α-GFP (from mouse) was used to detect MCA2[Y1344-]-GFP (expected MW of 187 kDa). Molecular weight standard is indicated in kDa. Full blots are shown in Appendix F.2. **D)** Live cell images of different developmental stages of knock-in cell line MCA2[Y1344Stop]-GFP. **E)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; kDa,

kilodaltons; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *white arrow* marks localization at cell periphery and vesicles; *red arrow* marks localization of MCA2^{Y1344Stop} at FV. scale bar, 5 µm.

4.1.4   *Pf*MCA2^{Y1344Stop} co-localizes with *Pf*Kelch13

Next, co-localization of the truncated *Pf*MCA2 and *Pf*Kelch13 was assessed to see whether the disruption influences the relative position of *Pf*MCA2 foci to *Pf*Kelch13 foci. For this the MCA2^{Y1344Stop}-GFP cell line was transfected with the episomally maintained *Pf*Kelch13-mCherry plasmid, resulting in the parasite line MCA2^{Y1344Stop}-GFP+K13 (Fig 14 A). Live cell imaging was performed by fluorescence microscopy and relative localization of *Pf*MCA2^{Y1344Stop} and *Pf*Kelch13 foci was quantified (Fig 14 B). Quantification showed that 59% of the *Pf*MCA2^{Y1344Stop} foci co-localized with *Pf*Kelch13, 20% of the foci showed partial co-localization and 21% of the *Pf*MCA2^{Y1344Stop} foci did not co-localize to *Pf*Kelch13 (Fig 14 B). *Pf*Kelch13 foci move in the living cell and particularly ring stages also change their position in the host cell (Grüring *et al.*, 2011). To reduce incorrect quantification of relative localization of foci due to movement of the cell and foci during image acquisition, smears of MCA2^{Y1344Stop}-GFP+K13 parasites were made, air-dried, and imaged via fluorescence microscopy. Quantification of the signal in smeared parasites revealed that the number of co-localizing foci increased to up to 72%, whereas 28% of the *Pf*MCA2^{Y1344Stop} foci did not co-localized with *Pf*Kelch13 foci (Fig 14 C).

In summary the results indicate that truncation of *Pf*MCA2 neither influences the localization of this protein, nor the relative localization of *Pf*MCA2 to *Pf*Kelch13.
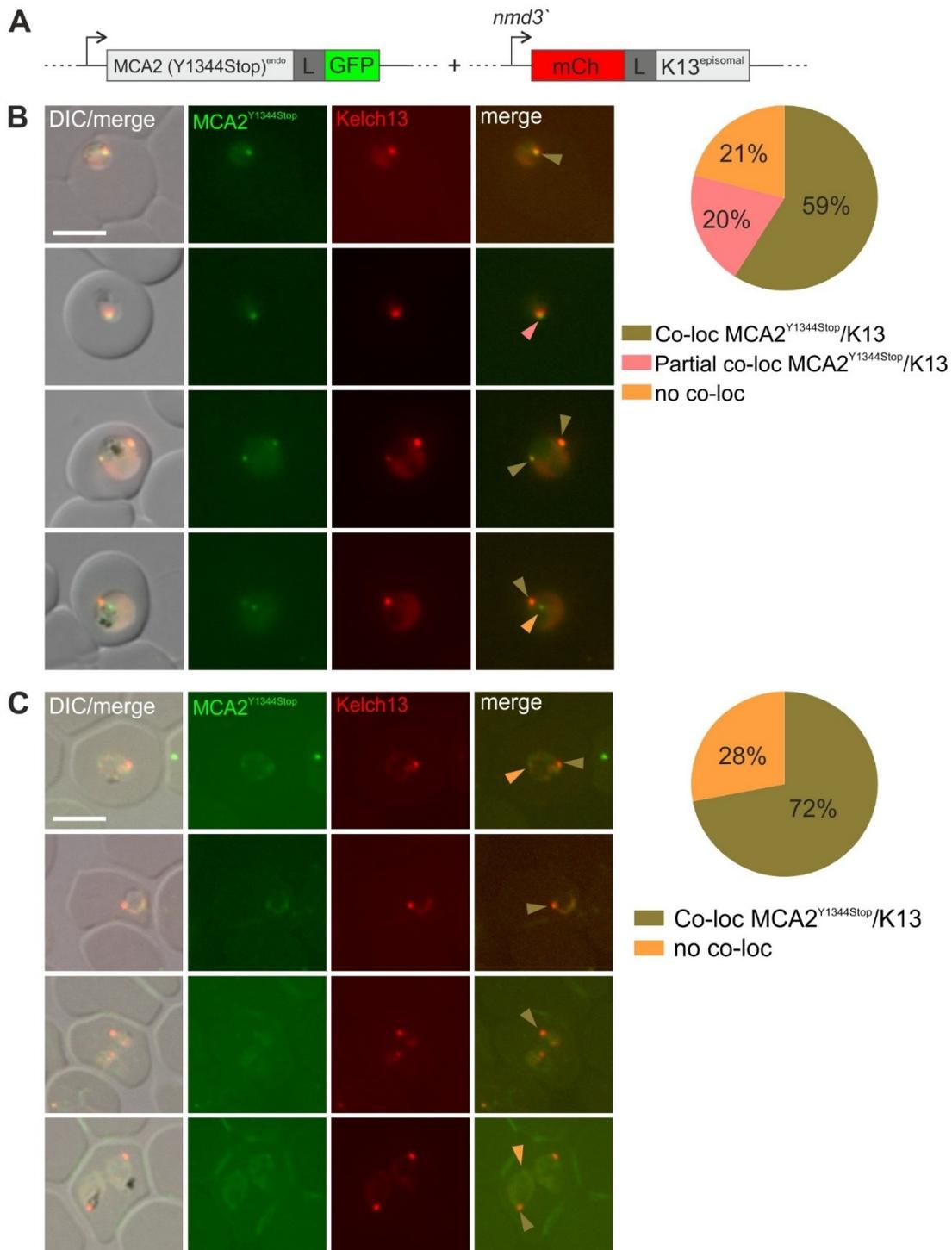
Figure 14) Relative localization of *Pf*MCA2^{Y1344Stop}-GFP foci to *Pf*Kelch13. A) Schematic of modified endogenous locus of MCA2^{Y1344Stop}-GFP parasites transfected with episomal mCherry-K13 plasmid. **B)** Live cell images of MCA2^{Y1344Stop}-GFP+K13 parasites and quantification of *Pf*MCA2^{Y1344Stop} and *Pf*Kelch13 foci and calculation of percentage co-localization (total co-localization, yellow color in the merge), partial co-localization (half of the foci size overlaps) and non-co-localization (close foci but no overlap or foci were further apart) *Pf*MCA2 and *Pf*Kelch13 foci. In total, three independent live cell imaging sessions were performed, and n = 46 cells were scored. **C)** Microscopy images of smeared, air-dried MCA2^{Y1344Stop}-GFP+K13 parasites and quantification of *Pf*MCA2^{Y1344Stop} and *Pf*Kelch13 foci and calculation of percentage co-localization, partial co-

localization, and non-co-localization foci (criteria same as in (B)). In total, two independent live cell imaging sessions were performed, and n = 20 cells were scored. *GFP*, green fluorescent protein; *mCh,* mCherry; *L,* linker; *DIC*, differential interference contrast; *green arrow* marks co-localizing foci, *pink arrow* marks partially co-localizing foci; *orange arrow* marks no co-localization between *Pf*MCA2^{Y1344Stop} and *Pf*Kelch13; scale bars, 5μm.

4.2 Functional characterization of *Pf*MyosinF (PF3D7_1329100, previously annotated as *Pf*MyosinC)

4.2.1 *Pf*MyosinF is found in close proximity to the Kelch13 compartment

DiQ-BioID and quantitative mass spectrometry revealed a series of potential interaction partners of *Pf*Kelch13 of which more than 10 were previously validated to co-located with Kelch13 (Birnbaum *et al.*, 2020). One of the hits that had not previously been validated was *Pf*MyosinF (previously annotated as *Pf*MyosinC). Myosins are motor proteins working with actin (Cheney *et al.*, 1993; Sellers, 2000). *Pf*MyosinF may therefore be a generator of force in the endocytosis process. Furthermore, mutations in *Pf*MyosinF were previously associated with ART resistance in genome wide association studies (Cerqueira *et al.*, 2017). For these reasons this potential *Pf*Kelch13 interactor was analyzed here.

To determine whether *Pf*MyosinF is located at *Pf*Kelch13 compartment and to assess its general cellular localization, native *Pf*MyosinF was tagged with 3xHA through modification of the endogenous *PfmyosinF* gene using SLI (Fig 15 A) (Birnbaum *et al.*, 2017). Correct integration of the construct mediating the corresponding genomic modification was confirmed by PCR (Fig 15 B). In a next step, the cell line expressing the 3xHA tagged wild type *Pf*MyosinF (MyoF^{wt}-3xHA) was transfected with an episomal mCherry-Kelch13 plasmid (MyoF^{wt}-3xHA+K13) (Fig 15 C). In order to assess the co-localization of both proteins, immune fluorescence assays were performed and the position of *Pf*MyosinF relative to *Pf*Kelch13 was determined in ring and trophozoite stage parasites. Here different fixation methods were applied, one that permits maximal antibody access but results in poor preservation of parasite morphology (acetone fixation) and one with less efficient antibody access but better cellular morphology (formaldehyde/glutaraldehyde fixation). For evaluation, the *Pf*MyosinF foci were observed in view of their relative position to *Pf*Kelch13 foci. Foci were defined as co-localizing when the totally overlapped, whereas partial co-localization was defined as foci which showed an overlap of 50%. Two foci were defined as close when they touched each other but showed no overlap or were not further apart than one focus size from each other. Foci were defined as non-colocalizing when they were further apart than one focus size.

In the parasites fixed with acetone, 80% of *Pf*MyosinF signal was found in foci and accumulations near to the FV (in 8 out of 10 evaluated hemozoin containing cells; see Appendix B.1) (Fig. 15 D). When the relative position of *Pf*MyosinF signal was compared to the *Pf*Kelch13 signal, it was observed that 16% of the *Pf*MyosinF foci co-localized with

*Pf*Kelch13 (Fig 15 E). Herein, 2 co-localization events (18%) out of 11 *Pf*MyosinF foci detected at the FV were counted (see Appendix B.1). 19% of *Pf*MyosinF foci showed a partial co-localization of foci, 63% were found close (foci touching, or not further apart than one focus size) and 2% of the *Pf*MyosinF foci were not proximal to *Pf*Kelch13 foci (Fig 15 E). These results indicate that *Pf*MyosinF rarely fully or partially co-localizes with *Pf*Kelch13 but is found in a close spatial arrangement with the *Pf*Kelch13 compartment.

To better preserve cellular morphology, parasites were also fixed in formaldehyde/glutaraldehyde and the position of *Pf*MyosinF foci in relation to *Pf*Kelch13 foci was evaluated again. Microscopy analysis of these cells showed that in 86% of cases (in 6 out of 7 hemozoin containing cells, see Appendix B.2) *Pf*MyosinF foci were located near the parasite FV as evident by proximity to the hemozoin signal observed in DIC (Fig. 15 F). Similar to acetone fixation, only a small proportion of *Pf*MyosinF foci fully or partially overlapped with *Pf*Kelch13, namely 17% and 10%. In contrast, 34% of *Pf*MyosinF foci were closely located to *Pf*Kelch13, while 38% did not show any co-localization (Fig 15 G). Analysis of the overlapping foci showed, that in ring stage parasites, 20% of total *Pf*MyosinF foci co-localized to *Pf*Kelch13, whereas in trophozoite stages, only 16% of all *Pf*MyosinF foci showed co-localization to *Pf*Kelch13 (see Appendix B.2). Since in ring stages it is not possible to analyze the relative position of foci to the FV, this was then investigated in trophozoite stages. Here, 30% of the co-localization events between *Pf*MyosinF and *Pf*Kelch13 (3 out of 10 *Pf*MyosinF FV-foci) were detected at the FV (see Appendix B.2).

In summary, both immune fluorescence assays indicate that *Pf*MyosinF foci show only limited overlap with the *Pf*Kelch13 compartment but that most of the *Pf*MyosinF foci are found in close proximity of the *Pf*Kelch13 compartment.
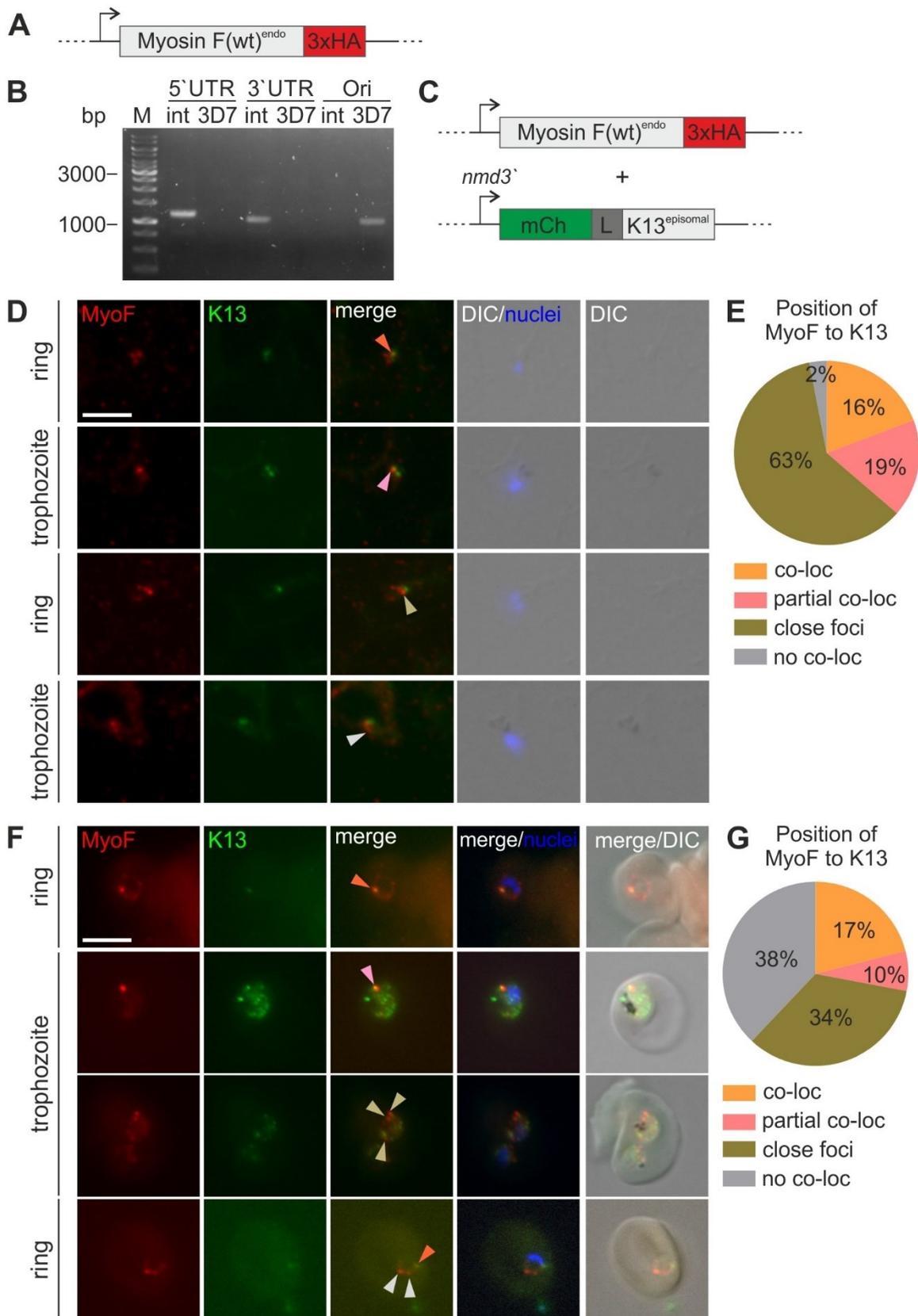
Figure 15) Localization of *Pf*MyosinF compared to *Pf*Kelch13. **A)** Schematic of the modified endogenous *Pf*MyosinF locus tagged with 3xHA. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain MyoF^wt^-3xHA

parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 1224 bp) and 3`-integration (3`UTR, 1093 bp) and to demonstrate absence of original locus in the MyoF^wt-3xHA parasites (Ori, 1037 bp). 3D7 gDNA was used as reference (ori 3D7 1037 bp). **C)** Schematic of modified endogenous locus of MyoF^wt-3xHA parasites transfected with episomal mCherry-K13 plasmid. **D)** Microscopy images of an IFA with acetone-fixed MyoF^wt-3xHA+K13 parasites. *orange arrow* marks overlapping/co-localizing *Pf*MyosinF and *Pf*Kelch13 foci; *pink arrow* marks partially overlapping/co-localizing foci; *green arrow* marks foci localizing close to each other; *grey* arrow marks foci with no overlap/co-localization. α-HA (from rabbit) was used to detect MyoF^wt-3xHA. α-RFP (from rat) was used to detect mCh-Kelch13 **E)** Quantification of spatial arrangement of *Pf*MyosinF and *Pf*Kelch13 foci. Shown is percentage of *Pf*MyosinF foci that co-localize (full overlap when all of the *Pf*MyosinF focus resulted in a yellow signal in the merge), partially co-localize (partial overlap of foci resulting in a yellow signal in the merging region), are closely located (foci touch or are not further apart than one focus size) or did not co-localize (foci further apart than one focus size). One aceton IFA was performed and n = 31 cells were scored. **F)** IFA images of formaldehyde/glutaraldehyde-fixed MyoF^wt-3xHA+K13 parasites. Arrow legends see (D). α-HA (from rabbit) was used to detect MyoF^wt-3xHA. α-RFP (from rat) was used to detect mCh-Kelch13. **G)** Quantification of *Pf*MyosinF and *Pf*Kelch13 foci (pie charts) as done in (E), n = 10 cells were scored from one IFA with this fixing method. *wt*, wild type; *endo*, endogenous; *HA*, hemagglutinin; *M*, marker (GeneRuler^TM 1 kb, Thermo Scientific); *bp*, base pair; *mCh,* mCherry; *L,* linker; *DIC*, differential interference contrast; scale bars, 5µm.

To assess the localization of *Pf*MyosinF and its relative positioning to *Pf*Kelch13 in living parasites, a cell line expressing *Pf*MyosinF tagged with 2xFKBP-GFP-2xFKBP from the endogenous locus (MyoF^wt-2x2) (kindly provided by Ernst Jonscher, cell line generated using SLI to modify *pfmyosinF* locus) was transfected with an episomal plasmid mediating expression of mCherry-Kelch13 (MyoF^wt-2x2+K13) (Fig 16 A). Live cell imaging with MyoF^wt-2x2+K13 parasites showed that in ring stages *Pf*MyosinF is only weakly expressed and was only detectable in older ring and trophozoite stage parasites (Fig 16 B). Quantification of co-localizing, partial co-localizing, close and non-co-localizing foci was performed as described for the IFAs before. This counting revealed that 8% of *Pf*MyosinF foci co-localized to *Pf*Kelch13, 12% showed a partial co-localization, 36% were closely located, while 44% of *Pf*MyosinF foci did not co-localized to *Pf*Kelch13 (Fig 16 C). Focusing especially on co-localization events of *Pf*MyosinF and *Pf*Kelch13 showed that 13% of the foci detected in late ring stages as well as in trophozoite stages co-localized at the FV confirming the results observed in acetone fixed MyoF^wt-3xHA+K13 parasites (see Appendix B.3). Comparing live cell imaging and immune fluorescence assays of MyoF^wt-3xHA+K13 revealed that formaldehyde/glutaraldehyde-fixed cells and live cell imaging achieved similar results with relatively high rates of non-co-localizing events, while acetone-fixed cells hardly detected non-co-localizing events.

In general, live cell imaging of the MyoF^wt-2x2+K13 parasites confirmed that *Pf*MyosinF is present at the Kelch13 compartment with some more than half of the *Pf*MyosinF foci overlapping either fully or partial with *Pf*Kelch13 or being closely located to it.
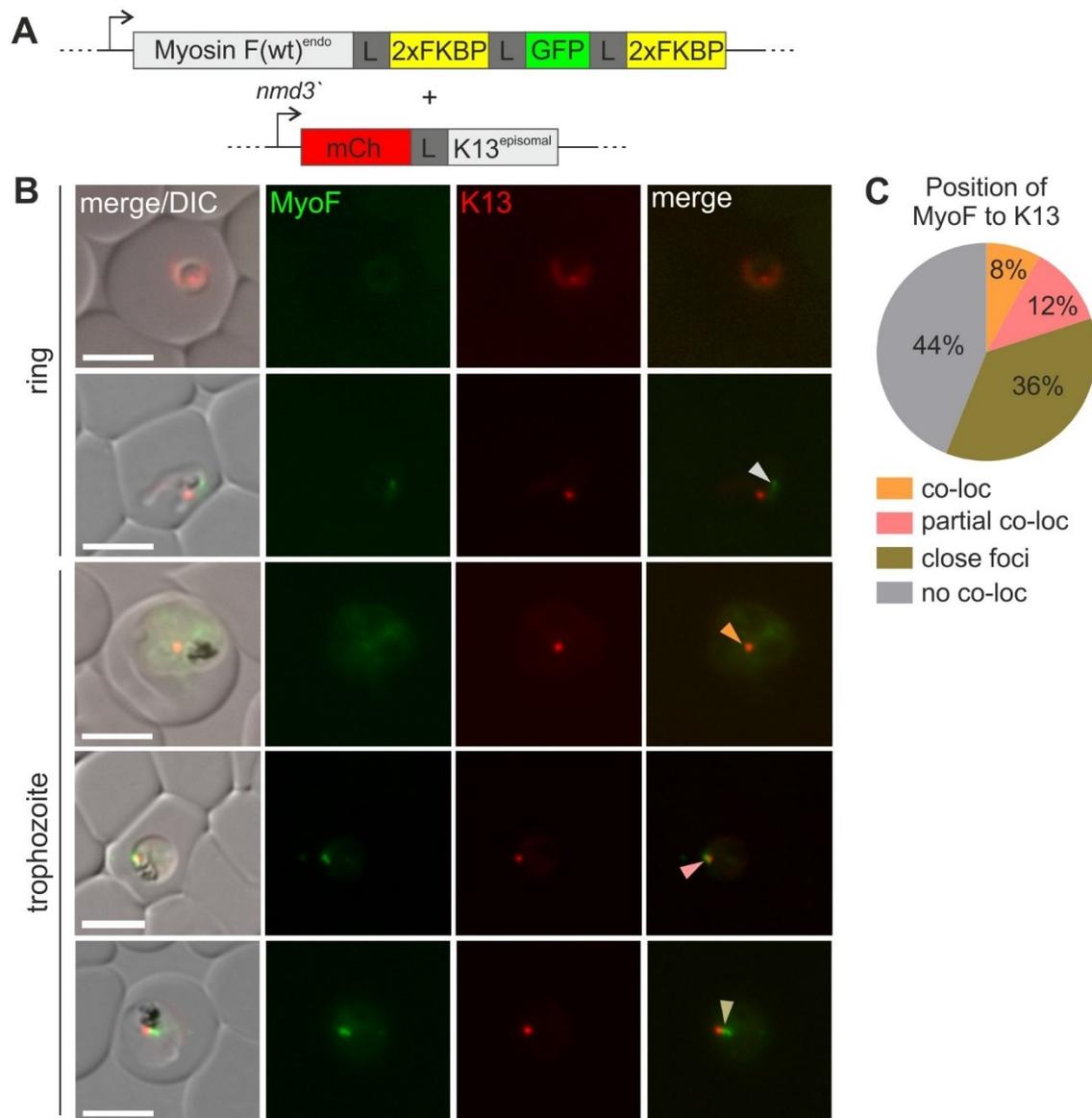
Figure 16) Relative localization of *Pf*MyosinF to *Pf*Kelch13. **A)** Schematic of modified endogenous locus of MyoF^wt^-2x2 parasites transfected with episomal mCherry-K13 plasmid. **B)** Live cell images of MyoF^wt^-2x2 + K13. *orange arrow* marks overlapping/co-localizing *Pf*MyosinF and *Pf*Kelch13 foci; *pink arrow* marks partially overlapping/co-localizing foci; *green arrow* marks foci localizing close to each other; *grey* arrow marks foci with no overlap/co-localization. **C)** Quantification of spatial arrangement of *Pf*MyosinF and *Pf*Kelch13 foci. Shown is percentage of *Pf*MyosinF foci that co-localize (full overlap when all of the *Pf*MyosinF focus resulted in a yellow signal in the merge), partially co-localize (partial overlap of foci resulting in a yellow signal in the merging region), are closely located (foci touch or are not further apart than one focus size) or did not co-localize (foci further apart than one focus size). Microscopy was performed on 3 independent days, n = 14 cells were scored. *wt*, wild type; *endo*, endogenous; *L,* linker; *FKBP*, FK506-binding protein; *GFP*, green fluorescent protein; *mCh,* mCherry; *DIC*, differential interference contrast; scale bars, 5µm.

4.2.2 Cytochalasin D treatment causes dissociation of *Pf*MyosinF and *Pf*Kelch13 in trophozoites

The compound Cytochalasin D is a toxin derived from fungi that binds to actin and inhibits its filamentous association, leading to the disruption of actin filaments. The data in section 4.2.1 indicated that *Pf*MyosinF is part of the *Pf*Kelch13 compartment and localizes close to or overlaps with *Pf*Kelch13. Since myosin belongs to the actomyosin complex, it was here tested whether treatment with the actin inhibitor Cytochalasin D based disruption of actin filaments causes a dissociation of *Pf*MyosinF and *Pf*Kelch13 signals. For this, the cell line MyoF^{wt}-2x2+K13 was treated with a final concentration of 10 µM Cytochalasin D for one hour and parasites were imaged using fluorescence microscopy directly at the start of the experiment (when Cytochalasin D was added, 0 h) and one hour after Cytochalasin D addition (Fig 17 A). In this experiment co-localization (total co-localization / overlap, yellow signal in the merge), partial co-localization (50% overlap of focus size, yellow signal in merging region) and no co-localization (foci touched but did not overlap or were further apart) of *Pf*MyosinF and *Pf*Kelch13 was quantified using slightly changed criteria (Fig 17 B). The analysis showed that due to poor signal intensity of *Pf*MyosinF foci in ring stage parasites of MyoF^{wt}-2x2+K13 (see also section 4.2.1) this analysis was inconclusive at that stage and therefore quantification was performed in early and late trophozoite stages. In early trophozoites, directly at the start of the experiment (0 h) the control cell line showed co-localizing and overlapping foci of both proteins congruent with that observed in the control cell line one hour after Cytochalasin D addition (1 hour without Cytochalasin D). In contrast, the same parasites treated with 10 µM Cytochalasin D for one hour showed no co-localization but partially overlapping foci, indicating the dissociation of *Pf*MyosinF and *Pf*Kelch13 foci in early trophozoites. In late trophozoite stages, a small proportion of co-localizing *Pf*MyosinF and *Pf*Kelch13 foci but no partial overlap was detected in the untreated control at the start of the experiment (0 h, time point when Cytochalasin D was added) and in the control one hour after Cytochalasin D addition (1 h without treatment). Here, in the parasites treated with 10 µM Cytochalasin D a small proportion of foci dissociated after one hour treatment reducing the amount of co-localizing *Pf*MyosinF and *Pf*Kelch13 foci. This indicates that in later trophozoites also a dissociation of *Pf*MyosinF and PfKelch13 is detectable. Additionally, in these stages also a dispersion of *Pf*MyosinF signal was seen (Fig 17 A, white asterisk).

In summary, these results indicate that the inhibition of actin polymerization using Cytochalasin D led to a reduction in the co-localization of *Pf*MyosinF and *Pf*Kelch13 foci in

early and late trophozoite stage parasites, and a dispersion of *Pf*MyosinF signal in late trophozoites indicating the dissociation of both proteins.
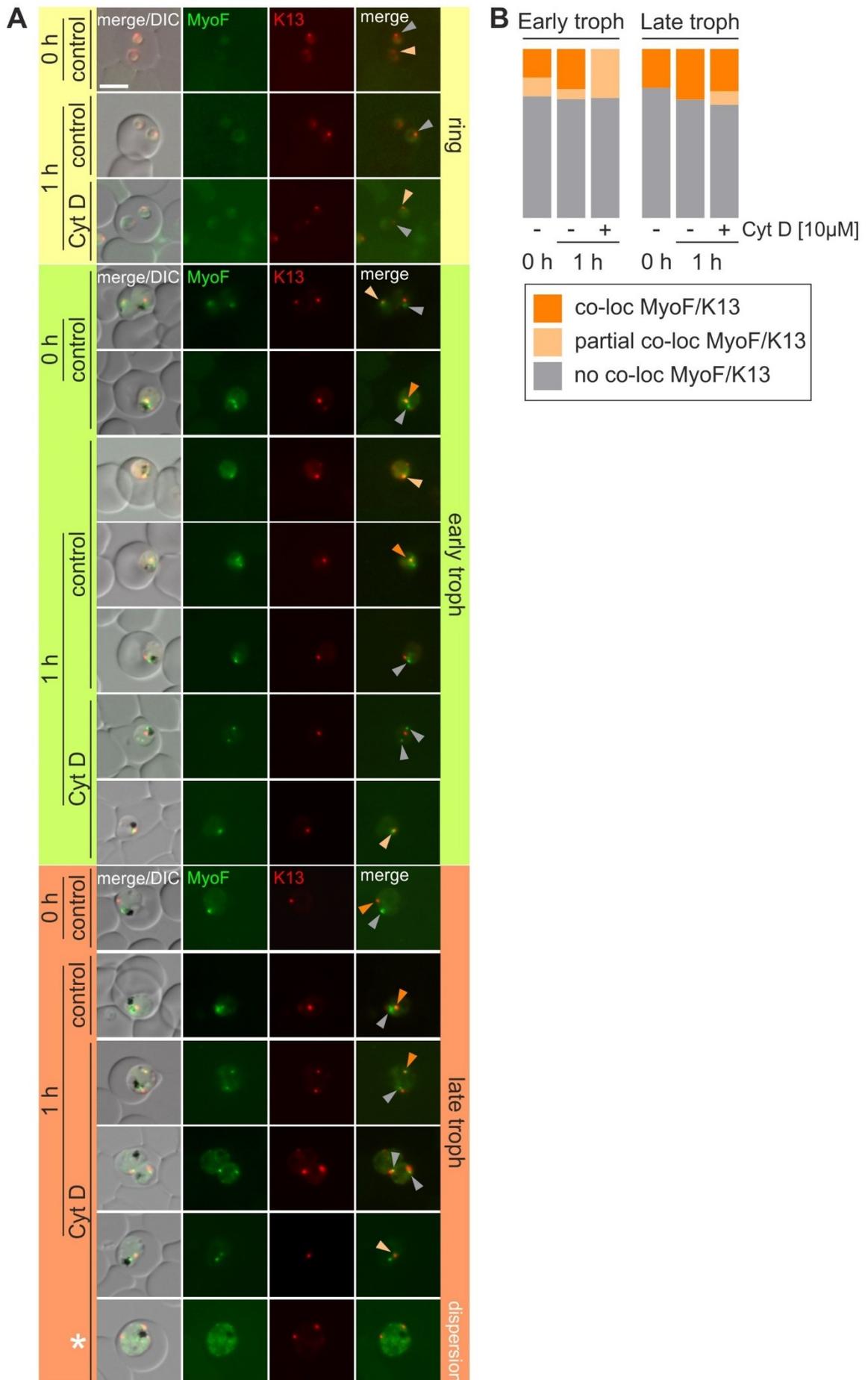
Figure 17) Cytochalasin D treatment leads to dissociation of *Pf*MyosinF and *Pf*Kelch13 foci in early and late trophozoites. **A)** Live cell images of MyoF^wt^-2x2+K13 parasites at different developmental stages. Images of control cell line and Cytochalasin D (10 µM) treated parasites were taken at the start of experiment (0 h) and one hour after treatment (1 h). *dark orange arrow* marks co-localizing *Pf*MyosinF and *Pf*Kelch13 foci; *light orange arrow* marks partially co-localizing foci; *grey arrow* marks foci with no overlap/co-localization; *white asterisk* marks dispersion of *Pf*MyosinF signal. **B)** Quantification of spatial arrangement of *Pf*MyosinF and *Pf*Kelch13 after Cytochalasin D treatment (calculations see Appendix B.5). Co-localization (total overlap, yellow color in merge), partial co-localization (50% overlap, yellow signal in merging region) and non-co-localization events (foci touch but do not overlap or are further apart). For start (0h), n = 17 cells, for the 1h control n = 13 and for Cytochalasin D-treated cells n = 20 cells were scored. *h*, hour; *DIC,* differential interference contrast; scale bar, 5µm.

4.3 *Pf*MyosinF is important for parasite development

4.3.1 Inactivation of *Pf*MyosinF leads to a reduced parasite growth

To assess the essentiality of *Pf*MyosinF for parasite development, a growth assay was performed after conditional inactivation of the protein using knock sideways. In a first step the mislocalization efficiency of the knock sideways was tested. For this, a previously generated cell line where the native *pfmyosinF* is endogenously tagged with *2xfkbp-gfp-2xfkbp* and episomally transfected with a 1xNLS mislocalizer was used (MyoF^wt^-2x2+1xNLS) (kindly provided by Ernst Jonscher). By adding rapalog, the inactivation of the target protein is induced via the mislocalization of the protein of interest into another cell compartment. Rapalog addition was continued for 22 hours in total and mislocalization was assessed at different timepoints via live cell imaging (Fig 18 A). Analysis of the data revealed that after one hour rapalog addition mislocalization of *Pf*MyosinF started but was still incomplete, while after two hours accumulation of the target protein at one specific area (due to NLS signal *Pf*MyosinF is targeted to the nucleus) increased although only partial mislocalization was observed and there was also *Pf*MyosinF that remained at its site of action. Observation of *Pf*MyosinF over 22 hours showed that the mislocalization was maintained but *Pf*MyosinF foci close to the food vacuole were still observed, indicating only incomplete mislocalization.

To investigate the impact of *Pf*MyosinF inactivation on parasite growth, a FACS-based growth assay was performed over 5 days (Fig 18 B). Asynchronous MyoF^wt^-2x2+1xNLS parasites were grown in the presence of rapalog for 5 days in total, and parasitemia was measured daily via flow cytometry. Simultaneously, a 3D7 control cell line was tested. This growth assay demonstrated that the partial inactivation of *Pf*MyosinF slightly impaired parasite growth but did not lead to parasite death. In contrast, 3D7 parasites grown in the

presence of rapalog did not show an altered growth compared to the control without rapalog. Quantification of relative growth on the last day of the experiment showed, that MyoF^wt-2x2+1xNLS cell line had a significant growth reduction of 25% compared to the same parasites grown without rapalog which was in contrast to the 3D7 control which showed no significant reduction in the culture grown with rapalog (Fig 18 C).

In summary, this data indicates that mislocalization of *Pf*MyosinF via knock sideways system is only partially successful but nevertheless significantly reduces parasite growth, indicating that this protein is important for parasite growth.
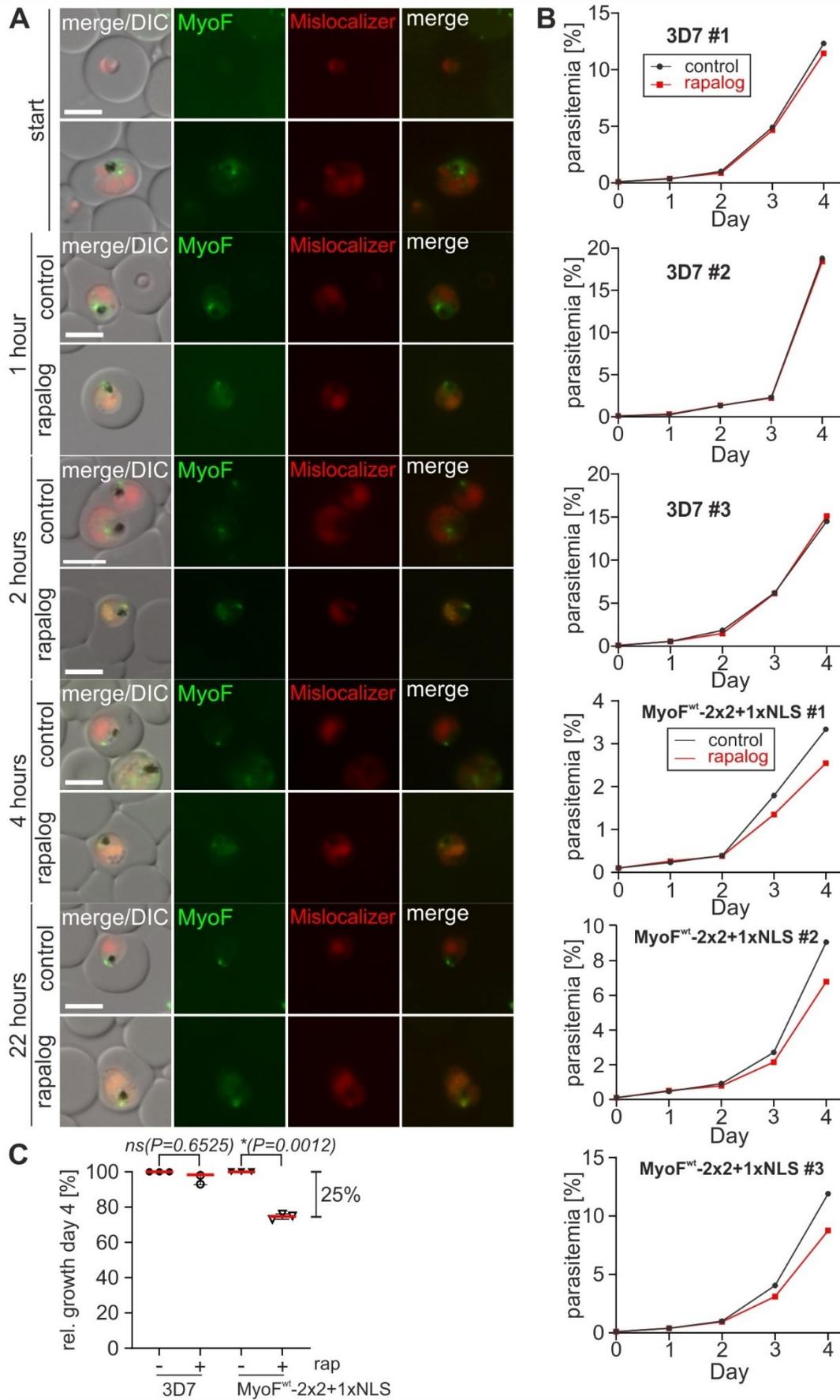
Figure 18) *Pf*MyosinF is important for parasite development. **A)** Live cell images of MyoF^wt^-2x2+1xNLS parasites. Control cell line and parasites cultured in the presence of rapalog (final concentration of 250nM) were imaged at different timepoints (indicated) after start of rapalog induction. **B)** Flow cytometry growth curves of asynchronous control and rapalog-treated 3D7 and MyoF^wt^-2x2+1xNLS parasites. Three independent experiments were conducted. **C)** Relative growth of rapalog treated 3D7 control and MyoF^wt^-2x2+1xNLS parasites at day 4 compared to respective untreated control. P value indicated, two-tailed, unpaired Welch`s t test. *DIC*, differential interference contrast; *NLS,* nuclear localization signal; *rap*, rapalog; scale bars, 5µm.

4.3.2 Inactivation of *Pf*MyosinF does not influence survival of ring stage parasites under ART treatment

The protein *Pf*MyosinF is part of the Kelch13 compartment and previous data showed that inactivation of *Pf*Kelch13 renders parasites resistant to ART. To test whether the inactivation of *Pf*MyosinF influences ART resistance, a standard RSA was carried out after the partial conditional inactivation of *Pf*MyosinF by knock sideways. Inactivation of *Pf*MyosinF was initiated either 12 hours or 24 hours prior to start of standard RSA and continued until one hour after the DHA pulse (Fig 19 A). Assessing the parasite survival rate 66 hours after DHA removal showed that inactivation of *Pf*MyosinF 12 hours prior to RSA start led to a survival rate of 0.27%, whereas a survival rate of 0.14% was reached when rapalog was added 24 hours prior to RSA start (Fig 19 B). The survival rate of the control (*Pf*MyosinF without rapalog) was even lower with 0.09% for rapalog addition 12 hours and 0.03% survival for rapalog addition 24 hours prior to the RSA. All survival rates were below the cut-off of 1% that defines resistance (Witkowski & Amaratunga *et al.*, 2013), indicating that inactivation of *Pf*MyosinF did not render parasite resistant to ART, although it should be noted that *Pf*MyosinF inactivation was only partial.
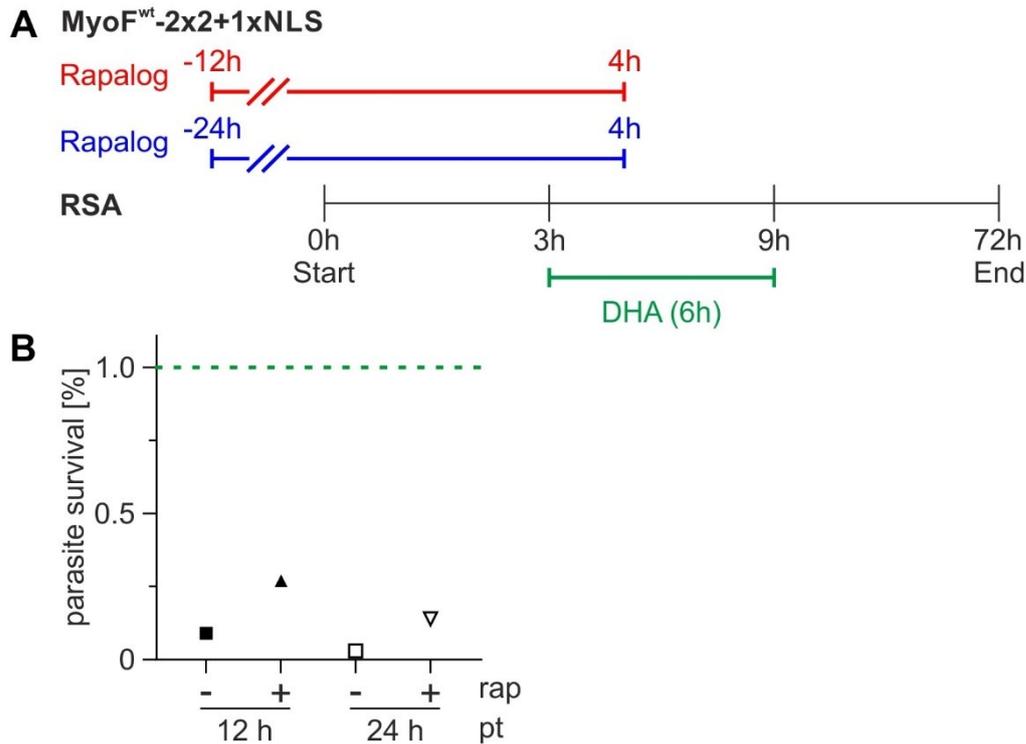
Figure 19) RSA of MyosinF^{wt}-2x2+1xNLS parasites after partial inactivation of *Pf*Myosin by knock sideways. **A)** Scheme showing timing of inactivation (by addition of rapalog) (red) 12 hours and (blue) 24 hours prior to RSA start and of RSA with MyosinF^{wt}-2x2+1xNLS parasites. **B)** Graph showing parasite survival rate in the RSA carried out as shown in (A). One experiment was performed per condition. *NLS*, nuclear localization signal; *h*, hour; *DHA*, dihydroartemisinin; *Rap*, rapalog; *red and blue line* indicates duration of rapalog treatment; *green line* indicates duration of DHA treatment; *green dotted line* marks cut-off value for resistance.

4.3.3   *Pf*MyosinF is involved in hemoglobin uptake

Previous experiments showed that *Pf*Kelch13 influences endocytosis of hemoglobin and subsequently affects ART resistance since ART gets activated by the degradation products of hemoglobin. This raises the question whether *Pf*Kelch13 compartment members like *Pf*MyosinF are also involved in hemoglobin uptake. To test this hypothesis, a bloated food vacuole assay was performed using the MyoF^{wt}-2x2+1xNLS cell line (kindly provided by Ernst Jonscher) to investigate if endocytosis was negatively affected when *Pf*MyosinF was inactivated. In this assay, the protease inhibitor E64 is used to block degradation of hemoglobin, leading to a bloated food vacuole phenotype when endocytosis is operational.

For this experiment, young trophozoites of 18 to 26 hours were treated with 33 µM E64 and rapalog was simultaneously added to one culture to induce inactivation of *Pf*MyosinF,

whereas the other culture served as a control without rapalog. After eight hours, the cells were stained with dihydroethidium and the number of cells showing a bloated food vacuole phenotype was scored using live cell imaging. Inactivation of *Pf*MyosinF under E64 treatment resulted in a reduction of the number of cells with a bloated food vacuole, while the control cells showed the typical bloated food vacuole phenotype (Fig 20 A). Quantification of the imaging data revealed that in two out of three experiments a significant reduction of bloated food vacuole phenotype was detected. On average, in all three experiments the untreated controls showed a mean proportion of 86% cells with bloated food vacuoles, whereas parasites grown in the presence of rapalog a mean proportion of 50% cells showed a significantly reduced bloated food vacuole phenotype (see Appendix B.4). Here, one representative experiment is depicted showing that 85% of control cells had bloated food vacuoles, while in rapalog cultivated parasites 32% of the cells developed a bloated food vacuole phenotype (Fig 20 B). Additionally, the number of vesicles was counted in the control and rapalog cell line resulting in a mean number of eight vesicles in the MyoF^{wt}-2x2+1xNLS parasites cultivated in the presence of rapalog, and a mean number of five vesicles in untreated the control cell line (Fig 20 C). This indicates that *Pf*MyosinF plays a role in endocytosis of hemoglobin.
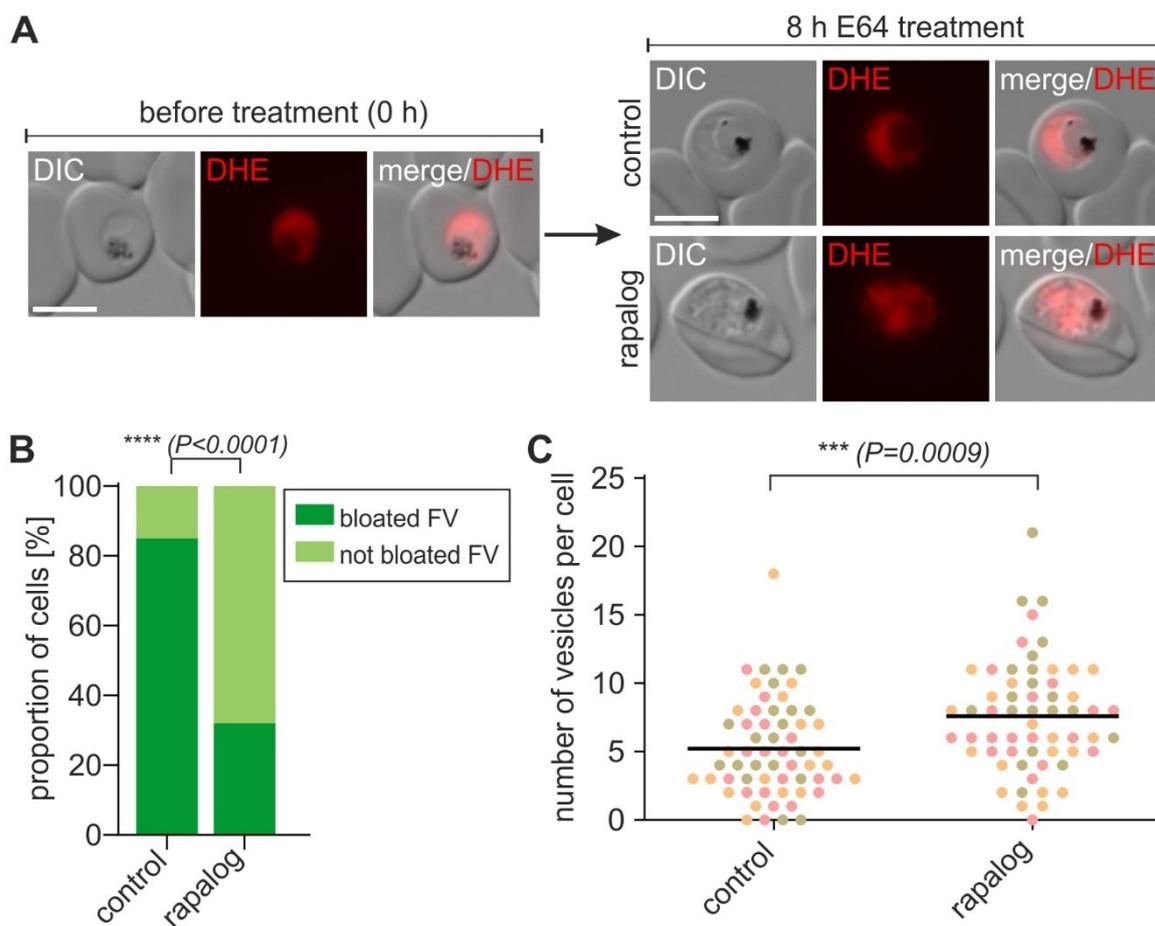
Figure 20) Inactivation of *Pf*MyosinF prevents hemoglobin uptake. Bloated food vacuole assay performed with MyoF^wt-2x2+1xNLS parasites eight hours after inactivation of *Pf*MyosinF. **A)** Live cell images of MyoF^wt-2x2+1xNLS parasites before addition of 33 µM E64 and rapalog (final concentration of 250nM) (left) and eight hours after rapalog addition (right). Parasite cytoplasm was stained with dihydroethidium (DHE) for imaging. Samples were blinded before imaging. **B)** Quantification of number of cells with bloated food vacuoles from (A). n = 34 cells were counted for each condition and Fisher`s exact test was performed; one representative of three independent experiments is shown (see Appendix B.4). **C)** Number of vesicles per cell of DIC images generated for (A) was quantified. n = 20 cells were evaluated in three independent experiments. P value is indicated, two-tailed, unpaired t test. *h*, hour; *DIC*, differential interference contrast; *DHE,* dihydroethidium; *FV*, food vacuole; scale bars, 5µm.

4.3.4   Actin filaments accumulate close to *Pf*MyosinF

The protein *Pf*MyosinF is part of the actomyosin motor. To test the relative position of *Pf*MyosinF to actin and monitor the assembly or disassembly of actin structures, the MyoF^wt-2x2 cell line (kindly provided by Ernst Jonscher) was transfected with an episomal plasmid containing a sequence encoding the actin-chromobody (a kind gift of Markus Meissner, (Periz *et al.*, 2017) linked to mCherry (MyoF^wt-2x2+chromobody) (Fig 21 A).

Live cell imaging was used to assess the location of *Pf*MyosinF and the actin-chromobody signal (Fig 21 B). Actin-chromobody was detected diffusely in the parasite cytosol probably referring to unbound chromobody since the marker is expressed in high levels by episomal plasmid (Periz *et al.*, 2017). Besides this diffuse pattern, actin-chromobody was also observed in filamentous structures at the cell periphery and in filaments shaping around vesicles. By evaluating the co-localization of *Pf*MyosinF and chromobody, the *Pf*MyosinF signal located next to the filamentous actin structures was scored, while the unbound cytosolic chromobody was not included. In ring stage parasites investigation of the relative position of *Pf*MyosinF to the chromobody was not possible since *Pf*MyosinF is very weakly expressed in this developmental stage (see also 4.2.1). In trophozoite stage parasites individual *Pf*MyosinF foci were detected that localized next to actin accumulations. Mostly, these closely localizing spots were observed close to the food vacuole but in some cases *Pf*MyosinF and actin accumulations were found at vesicles (as determined based on the DIC image) that were not proximal to the food vacuole. A total co-localization of the target protein with actin-marker was not detected.

In summary, this data indicates that *Pf*MyosinF is detected next to the food vacuole and vesicular structures and filamentous actin is accumulating close to *Pf*MyosinF foci.
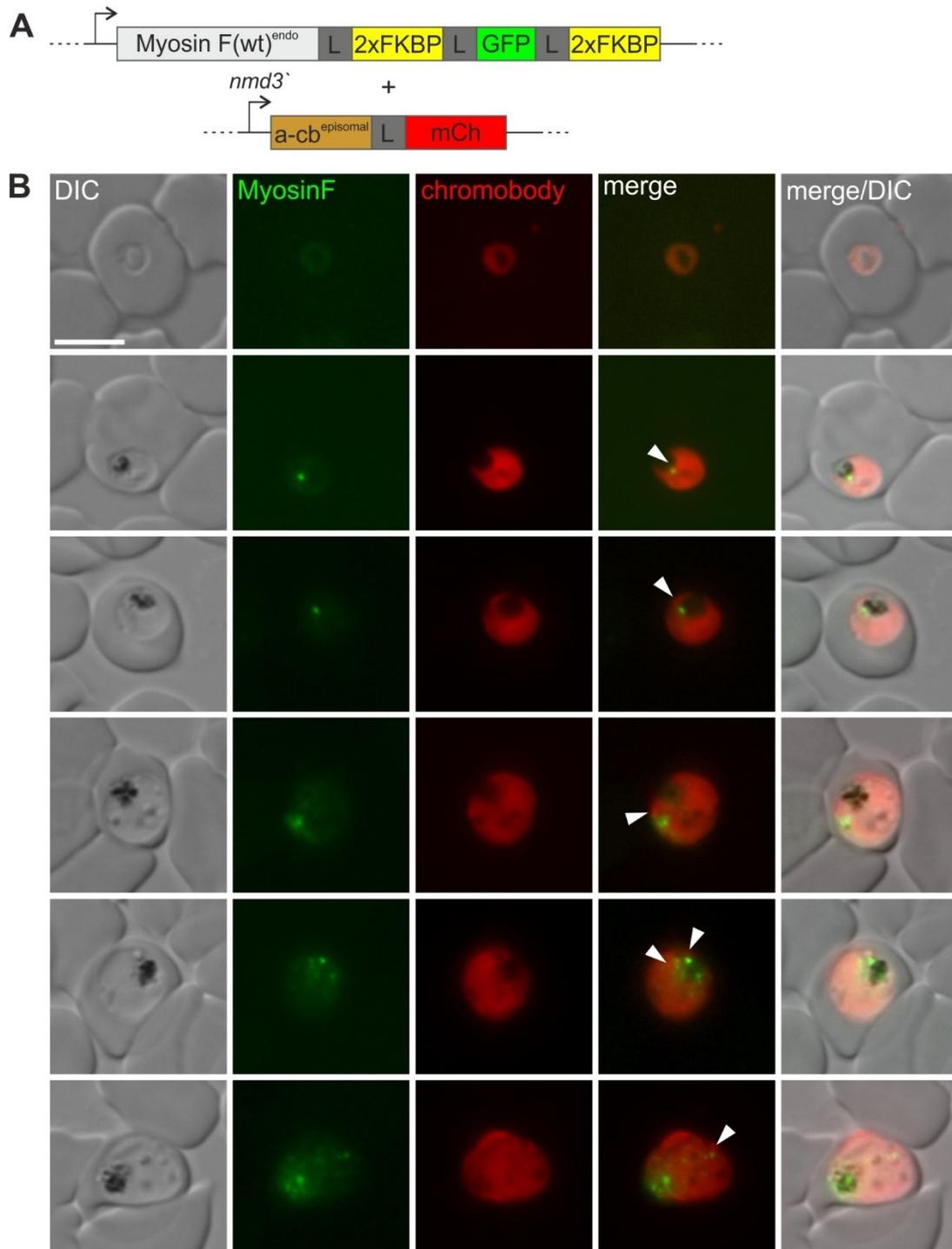
Figure 21) Actin filaments accumulate next to *Pf*MyosinF. **A)** Schematic of modified endogenous locus of MyoF^wt-2x2 parasites transfected with episomal actin-chromobody fused to mCherry. **B)** Live cell images of MyoF^wt-2x2+chromobody parasites. *wt*, wild type; *L,* linker; *FKBP*, FK506-binding protein; *GFP*, green fluorescent protein; *a-cb*, actin-chromobody; *mCh,* mCherry; *DIC*, differential interference contrast; *white arrow* marks accumulation of actin; scale bar, 5µm.

4.3.5 Inactivation of *Pf*MyosinF increases co-localization with PI3P-positive structures at the food vacuole

Phosphatidylinositol 3-phosphate (PI3P) is a hallmark of early endosomes in eukaryotic cells and was previously detected at host cell cytosol filled vesicles when their transport to the food vacuole was prevented (Jonscher *et al.*, 2019). For these experiments, PI3P was detected using an mCherry tagged marker called P40PX, a widely used marker to detect this phosphoinositide (Balla, 2013). To investigate whether *Pf*MyosinF is important during endolysosomal pathway, and the knock sideways induced vesicles have endosomal character, the cell line MyoF^wt-2x2 (kindly provided by Ernst Jonscher) was transfected with an episomal plasmid containing an expression cassette encoding 1xNLS-FRB-T2A-P40PX-mCherry, resulting in the parasite line MyoF^wt-2x2+1xNLS-P40PX. If *Pf*MyosinF vesicles have endosomal character, the vesicles induced by knock sideways were expected to co-localize with P40PX signal, as this is a marker of endosomal vesicles.

The MyoF^wt-2x2+1xNLS-P40PX cell line was grown in the presence of rapalog for 24 hours to induce inactivation of *Pf*MyosinF and live parasites were imaged at different time points (Fig 22 A). As expected (Tawk *et al.*, 2010; Boddey *et al.*, 2016), the P40PX marker predominantly labelled the food vacuole membrane or foci in proximity of the food vacuole and additionally, vesicular structures at cell periphery (Fig. 22 A). *Pf*MyosinF was mainly detected in foci close to the food vacuole and was also partially refractory to mislocalization up to 24 hours when rapalog was added. This is consistent with previous findings in section 4.3.1. The relative position of *Pf*MyosinF to the P40PX marker was scored (Fig. 22 B). Here, co-localization was defined as total overlapped of *Pf*MyosinF with P40PX foci or accumulations (yellow color in the merge), while *Pf*MyosinF was defined as closely located to P40PX when the foci touched but did not overlap or were not further apart than one focus size. No-colocalization was detected, when *Pf*MyosinF and P40PX were further apart than one focus size. Scoring was performed in trophozoite stage parasites since the *Pf*MyosinF signal is weakly expressed in ring stages (Fig 22 A; see also section 4.2.1). Quantification showed that at the start of the experiment when rapalog was added 70% of the *Pf*MyosinF foci were closely located to P40PX, and 30% of the foci co-localized to P40PX (see Appendix B.6). Similar results were seen in the control grown without rapalog after two hours. In contrast, in the MyoF^wt-2x2+1xNLS-P40PX parasites cultivated in the presence of rapalog for two hours 91% of the *Pf*MyosinF foci co-localized to P40PX indicating an increase of co-localization events upon the inactivation of *Pf*MyosinF. Quantification of the samples taken after four hours, and 24 hours revealed similar results compared to the scoring after two hours confirming that inactivation of *Pf*MyosinF promotes the co-

localization with P40PX. These results were rather surprising, as due to the 1xNLS signal, *Pf*MyosinF should be targeted to the nucleus and co-localization with P40PX should be manly detected there. One option for the localization at the food vacuole could be, that the skip peptide between NLS-FRB and P40PX-mCherry is not fully skipped. This could result in the binding of the FKBP domains at *Pf*MyosinF to the FRB on P40PX, recruiting the *Pf*MyosinF to the P40PX structures.

In summary, the data indicates that *Pf*MyosinF is closely located to PI3P positive structures represented by the P40PX marker. Upon addition of rapalog, which causes partial mislocalization of *Pf*MyosinF, co-localization between P40PX and non-mislocalized *Pf*MyosinF increased and was mainly detected at that food vacuole, since *Pf*MyosinF localizes close to the food vacuole and was refractory to mislocalization. This preliminary experiment was performed to assess the relative position of *Pf*MyosinF and P40PX. Because the mislocalization was only partial, and the skipping of the episomal construct is potentially insufficient, the data need to be confirmed by additional experiments.
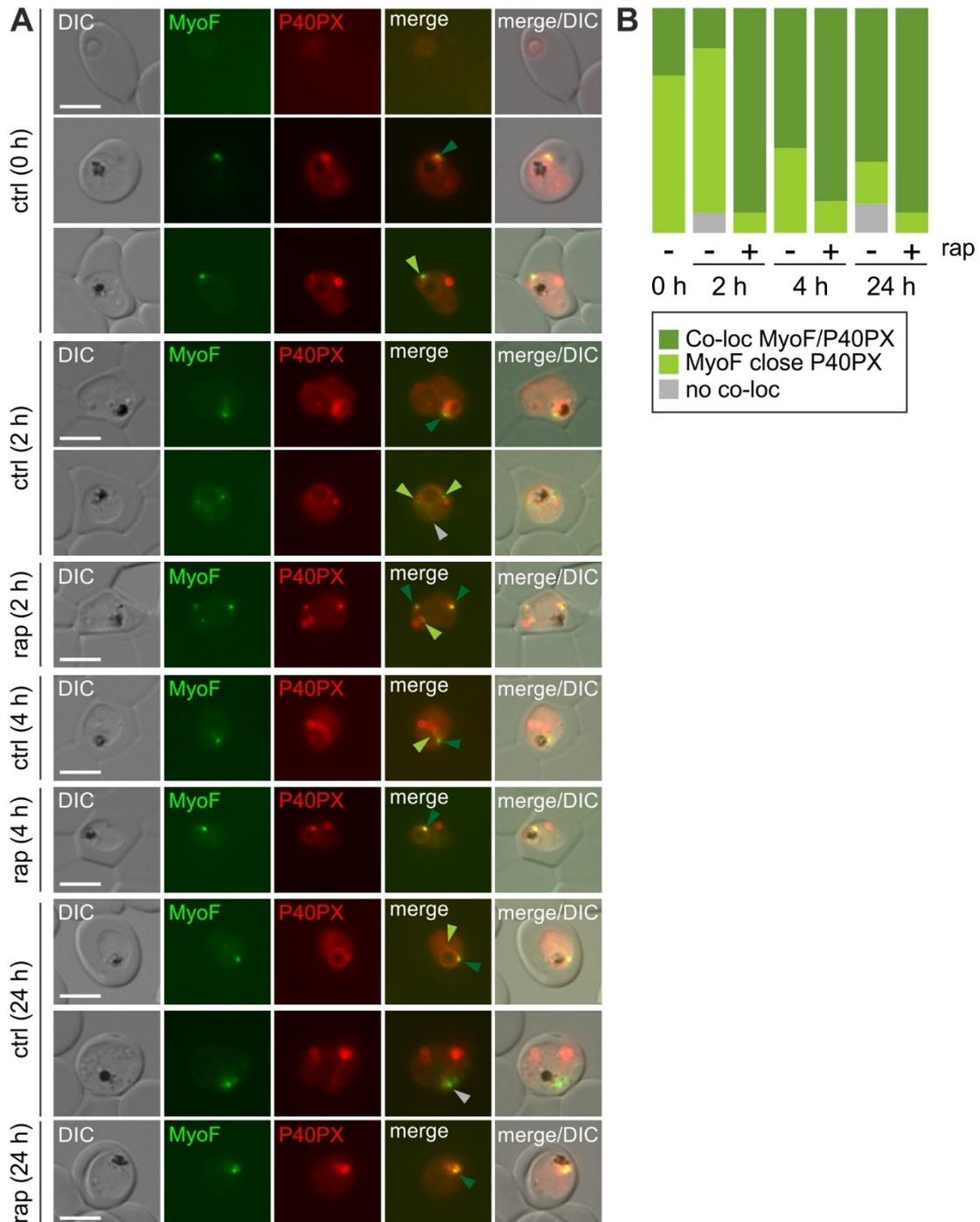
Figure 22) Inactivation of *Pf*MyosinF leads to increased co-localization with PI3P-positive structures (P40PX marker) at the food vacuole. **A)** Live cell images of MyoF^wt^-2x2+1xNLS-P40PX parasites at different time points after rapalog induction. Images of control cell line and parasites grown in the presence of rapalog (final concentration of 250nM) were taken at start of experiment and 2 h, 4 h and 24 hours after induction with rapalog. *Dark green arrow*, marks co-localization of *Pf*MyosinF and P40PX; *light green arrow*, marks foci closely located, *grey arrow*, marks, no co-localization. **B)** Quantification of *Pf*MyosinF and PI3P-positive foci (P40PX) and calculation of co-localization of *Pf*MyosinF and PI3P-positive structures (total overlap of foci, yellow signal in the merge), close localization (foci are touching but not overlapping or foci are not further apart than one focus size) and non-co-localization events (foci are further apart than one focus size) (calculations see Appendix B.6). For start n = 8 cells, for 2 hours n = 7 (control) and n = 12 cells (rapalog), for 4

hours n = 4 (control) and n = 11 cells (rapalog) and for 24 hours n = 10 (control) and n = 6 cells (rapalog) were scored. *Ctrl*, control (no rapalog induction); *rap*, rapalog (250nM final concentration); *h*, hour; *DIC,* differential interference contrast; *P40PX*, marker for PI3P-positive structures; scale bars, 5µm.

4.4 *Pf*Kelch13 compartment members are involved in ART resistance

As the *Pf*Kelch13 compartment contains other essential proteins involved in endocytosis (see section 4.3.3) it was here tested, if the inactivation of these proteins, namely *Pf*UBP1 (PF3D7_0104300), *Pf*Eps15 (PF3D7_1025000), *Pf*KIC7 (PF3D7_0813000), and *Pf*AP-2µ (PF3D7_1218300), as well as inactivation of *Pf*Kelch13 (PF3D7_1343700) itself lead to ART resistance.

For inactivation of the candidates, the knock sideways system was used (Birnbaum *et al.*, 2017). The time of induction of inactivation prior to start of the RSA, as well as the duration of keeping rapalog during the test, was adjusted to the knock sideways efficiency and its effect on survival for each of the respective candidates. This was done to assure the successful inactivation of the protein, while at the same time avoiding a severe loss of viability of the parasites.

4.4.1 *Pf*Kelch13 mediated ART resistance

In a first step, we tested whether inactivation of *Pf*Kelch13 (PF3D7_1343700) itself changes susceptibility to ART. For this, a standard *in vitro* RSA was performed with the 3xNLS *Pf*Kelch13 knock sideways parasites (Kelch13^{wt}+3xNLS), a line that permits partial inactivation of *Pf*Kelch13 without loss of parasite viability (Birnbaum *et al.*, 2017). Partial inactivation of *Pf*Kelch13 was initiated 12 hours prior to the start of the RSA through addition of rapalog and continued until ART was removed (Fig 23 A) and by this, parasites became resistant to ART as evident by a mean survival rate of 27.20%, (SD, +/- 8.05) (Figure 23 C). In contrast, the 3D7 control cell line, subjected to the same rapalog-addition regimen prior to the RSA, showed a mean survival rate of 0.08% (SD, +/- 0.05) (Fig 23 B, C).
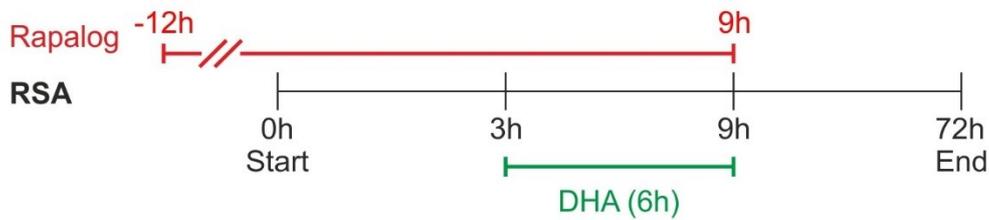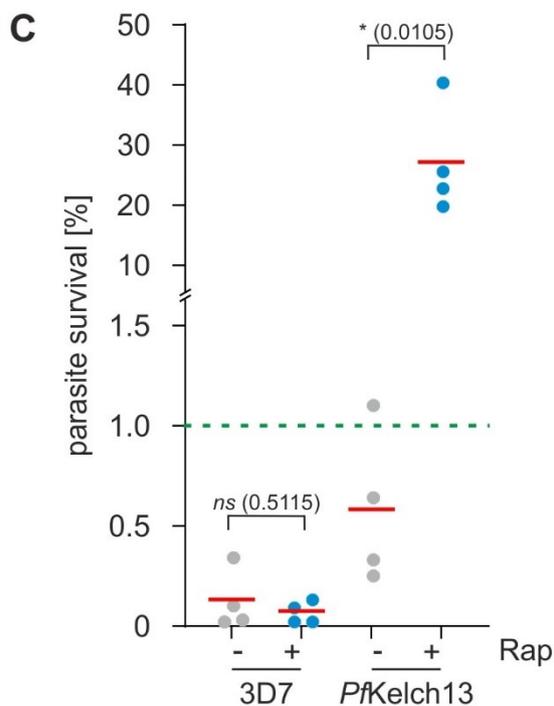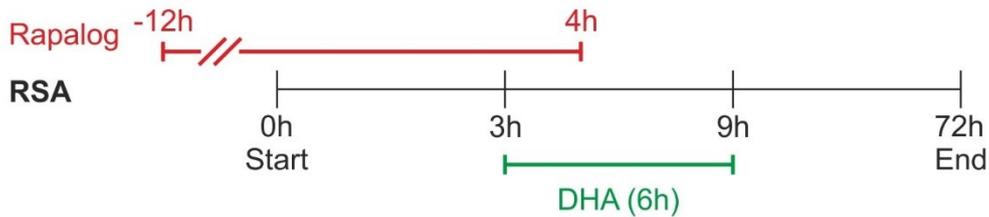
Figure 23) RSA of Kelch13^{wt}+3xNLS parasites after partial inactivation of *Pf*Kelch13 by knock sideways. A, B) Scheme showing timing of inactivation (by addition of rapalog) and of RSA with Kelch13^{wt}+3xNLS (A) and with 3D7 (B) control parasites. **C)** Graph showing parasite survival rate 72 hours after start of the RSA carried out as shown in A) and B). Four independent experiments were performed. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch´s t test. *NLS*, nuclear localization signal; *h*, hour; *DHA*, dihydroartemisinin; *Rap*, rapalog; *red line* indicates duration of rapalog treatment; *green line* indicated duration of DHA treatment; *green dotted line* marks 1% cut-off value for resistance (Witkowski & Amaratunga *et al.*, 2013).

4.4.2   *Pf*Kelch13-independent ART resistance

Next, RSA were carried out after the essential *Pf*Kelch13 compartment proteins were partially inactivated by knock sideways.

For *Pf*UBP1 (PF3D7_0104300) rapalog, to induce the knock sideways and inactivate the protein, was added one hour prior to the ART pulse and was removed one hour after addition of ART (Fig 24 A). This partial inactivation resulted in a survival rate of 2.00% (SD, +/- 0.62), indicating that the parasites became resistant to ART to a moderate level (Fig 24 D). The next candidate, *Pf*AP-2µ (PF3D7_1218300), was inactivated seven hours before the RSA start and rapalog treatment was continued until one hour after the addition of ART (Fig 24 B). Conditional removal of *Pf*Ap-2µ using knock sideways rendered parasite resistant to ART with a mean survival rate of 2.16% (SD, +/- 0.59) (Fig 24 D), again resulting in moderate levels of survival. For *Pf*Eps15 (PF3D7_1025000), efficient inactivation required addition of rapalog 18 hours before starting the RSA and rapalog treatment was maintained until one hour after ART addition (Fig 24 C). This resulted in a mean survival rate of 2.29% (SD, +/- 1.55), comparable to the results obtained with *Pf*UBP1 and *Pf*AP-2µ (Fig 24 D). The highest resistance level was measured for *Pf*KIC7 (PF3D7_0813000) that was inactivated the same way as *Pf*UBP1, which resulted in a mean survival rate of 12.31% (SD, +/- 1.53). In contrast, addition of rapalog to 3D7 control cell line 12 hours prior to start of the RSA (Fig 23 B), as well as inactivation of *Pf*2102 (PF3D7_0210200) seven hours prior to start (Fig 24 B), an unrelated control protein that is not part of the *Pf*Kelch13 compartment but is essential for parasite growth (Birnbaum *et al.*, 2017), did not result in detectable resistance as measured by RSA. The 3D7 control cell line achieved a mean survival rate of 0.08% (SD, +/- 0.05) (Fig. 24 D), while *Pf*2102 showed a survival rate of 0.16% (SD, +/- 0.09) after inactivation of the target protein (Fig. 24 B, D).

In summary, these results show that partial inactivation of essential *Pf*Kelch13 compartment proteins, all of which are involved in endocytosis, as well as inactivation of *Pf*Kelch13 itself, reduces the responsiveness of *P. falciparum* parasites to ART. These finding indicate that these proteins are all part of the resistance pathway.
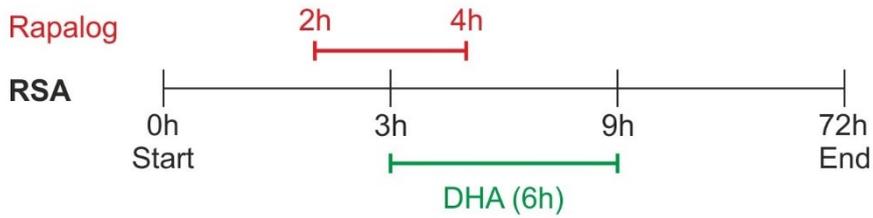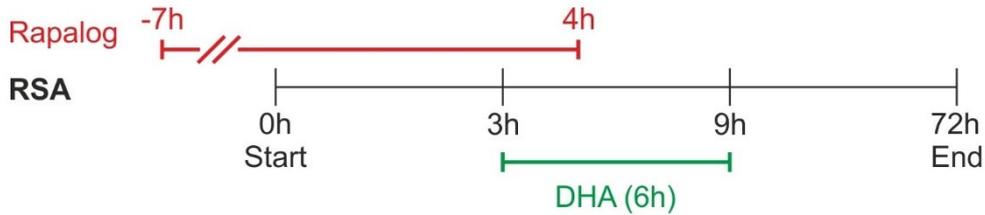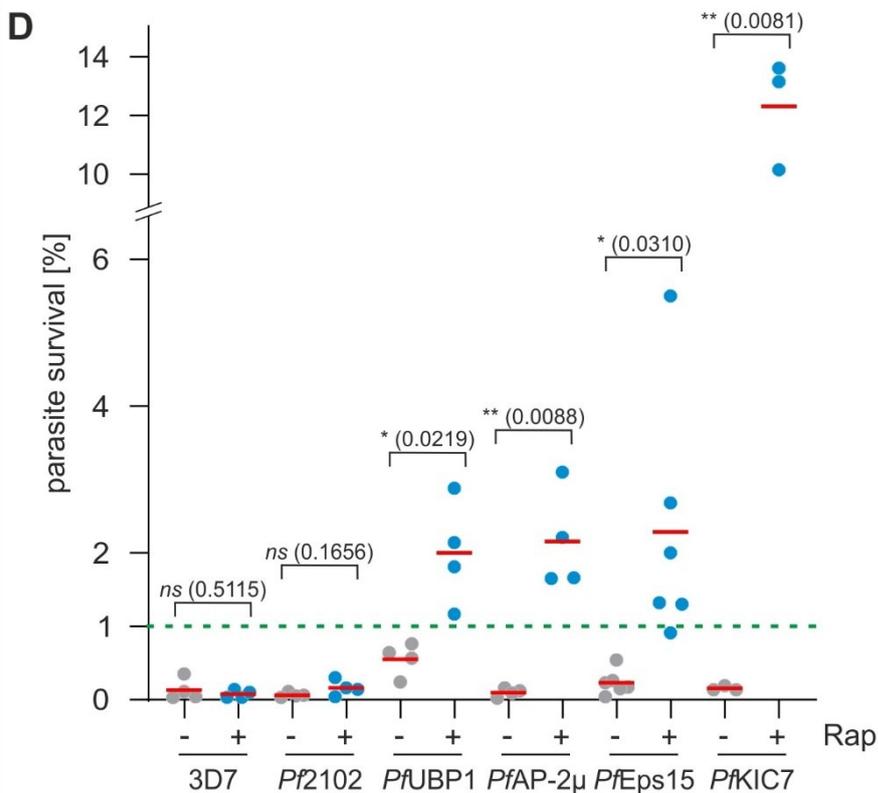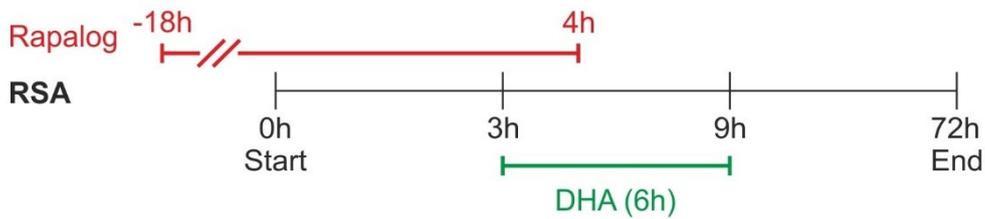
Figure 24) RSA of essential *Pf*Kelch13-compartment members after partial inactivation of respective candidate by knock sideways. **A, B, C)** Scheme showing timing of inactivation (by addition of rapalog) and of RSA with *Pf*UBP1+1xNLS and *Pf*KIC7+1xNLS (A), with *Pf*AP-2μ+1xNLS

and *Pf*2102+lyn (B) and *Pf*Eps15+1xNLS (C). **D)** Graph showing parasite survival rate 72 hours after start of the RSA carried out as shown in (A), (B) and (C). Scheme of rapalog induction for 3D7 control parasites is depicted in Fig. 23 B. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch´s t test. *NLS*, nuclear localization signal; *h*, hour; *DHA*, dihydroartemisinin; *Rap,* rapalog; *red line* indicates duration of rapalog treatment; *green line* indicated duration of DHA treatment; *green dotted line* marks 1% cut-off value for resistance (Witkowski & Amaratunga *et al.*, 2013).

4.5 Mutations in Africa and generation of mutation pools

4.5.1   Selection of SNPs for mutation pools

To test whether mutations in *Pf*Kelch13 compartment members could be responsible for ART resistance in the field, different non-synonymous SNPs in *pfkic1* (PF3D7_0606000), *pfkic2* (PF3D7_1227700), *pfkic4* (PF3D7_1246300), *pfkic5* (PF3D7_1138700), *pfkic7* (PF3D7_0813000), *pfkic9* (PF3D7_1442400), *pfmyosinF* (PF3D7_1329100), *pfubp1* (PF3D7_0104300), identified in field samples were chosen and inserted into 3D7 parasites to test their relevance for ART resistance.

To search for new mutations, the following sources were used. First, from the MalariaGEN *Plasmodium falciparum* Community Project (MalariaGEN *et al.*, 2021), non-synonymous mutations were included for testing when they were mainly present in Africa with low to medium prevalence (0.1% to 40%). Furthermore, after personal communication with Oumou Maiga-Ascofaré, six mutations found in Southeast Asia were also added to the list. Overall, this resulted in 125 SNPs in 8 genes (Table 1).

In addition, mutations in these genes found in a sample obtained during the Fever without Source study in Ghana (Hogan *et al.*, 2018) and derived from a patient that was visiting the ward repeatedly, suffering from malaria were included. The person was treated several times with ART and sequencing confirmed that the patient was always infected with the same *Plasmodium* strain, indicating an infection with an ART resistant strain (Oumou Maiga-Ascofaré, personal communication). However, no mutation was present in *pfkelch13*, indicating *pfkelch13*-independent resistance. To investigate whether mutations in any of our genes of interest was the reason for this apparent resistance, genomic DNA was extracted from blood samples of this patient and our candidate genes were sequenced. This sequencing included *pfubp1*, *pfmyosin F*, *pfkic6*, *pfkic7*, *pfap-2µ*, *pfeps15* and parts of *pfap-2α* and *pfmca2* (DNA extraction, PCR, sequencing, and preparation of samples for external sequencing at Microsynth Seqlab was kindly performed by Birgit Förster). This

revealed 18 non-synonymous mutations and 8 synonymous mutations (compared to 3D7 used as a reference), of which 7 non-synonymous and 6 synonymous mutations overlapped with SNPs also listed in MalariaGEN, and 11 non-synonymous and 2 synonymous mutations that had not been described before (Appendix D.1). Beside *pfkelch13*, no mutations were found for *Pfkic7* and *Pfap-2µ*. Overall, this resulted in 7 non-synonymous SNPs (including two mutations in *Pf*MyosinF that had not been described before) in 3 genes that were selected for analysis together with the mutations selected from the database searches. The remaining 11 non-synonymous SNPs found in the sequencing were not tested in the pools since either the N-terminal location of the SNPs in very huge genes hindered their inclusion (i.e.for *Pf*UBP1) or sequencing of the candidates was not finished until the pools were prepared and synthesized by GenScript (i.e. *Pf*MyosinF).

To make possible to analyze this large number of mutations, the mutations were tested in pools (i.e. inserting all SNPs at the same time into the gene) in 3D7 using SLI to modify the corresponding genes (Table 1).

Table 1) List of all SNPs collected for mutation pools of the candidate genes indicated. Detailed description of the selected mutations is provided in the respective section of each candidate. Data on prevalence of the different candidates are listed in Appendix D.1. *Asterisk (*)*, indicates SNP that were not tested in the pool since they were detected after SLI plasmids were prepared for transfection or were located in the N-terminal region of the gene which would have complicated their inclusion due to the large size of this gene; *asterisk (**)*, mark synonymous mutations that were not included in the testing.

Candidate	Mutation	Reference
*Pf*KIC1 (PF3D7_0606000)	D483H	MalariaGen
	D535G	MalariaGen
	D862V	MalariaGen
	I873M	MalariaGen
	K231E	MalariaGen
	K676N	MalariaGen
	K883T	MalariaGen
	N179K	MalariaGen
	N633Y	MalariaGen
	N634Y	MalariaGen
	N688Y	MalariaGen
	N971S	MalariaGen
	Q1042K	MalariaGen
	S394N	MalariaGen
	S429P	MalariaGen
	T679A	MalariaGen
	V543F	MalariaGen

PfKIC2 (PF3D7_1227700)	E207Q	MalariaGen
	E343K	MalariaGen
	G167D	MalariaGen
	K321N	MalariaGen
	K651M	MalariaGen
	N449T	MalariaGen
	Y472F	MalariaGen
PfKIC4 (PF3D7_1246300)	D120N	MalariaGen
	I121V	MalariaGen
	M375V	MalariaGen
	N123S	MalariaGen
	N405S	MalariaGen
	N624I	MalariaGen
	N661H	MalariaGen
	P170S	MalariaGen
	P621A	MalariaGen
	T888A	MalariaGen
	V619A	MalariaGen
	Y179C	MalariaGen
PfKIC5 (PF3D7_1138700)	A471T	MalariaGen
	D169N	MalariaGen
	E1055G	MalariaGen
	E1354D	MalariaGen
	E1646G	MalariaGen
	E497Q	MalariaGen
	F570L	MalariaGen
	G1417D	MalariaGen
	G264D	MalariaGen
	G727E	MalariaGen
	H1198Y	MalariaGen
	H138Y	MalariaGen
	K1411R	MalariaGen
	K258R	MalariaGen
	K384I	MalariaGen
	K751Q	MalariaGen
	K985N	MalariaGen
	L1066P	MalariaGen
	M324V	MalariaGen
	M975I	MalariaGen
	N1268H	MalariaGen
	N525S	MalariaGen
	P516S	MalariaGen
	Q110E	MalariaGen
	Q1127K	MalariaGen
	Q1421K	MalariaGen
	R1040K	MalariaGen
	S1044R	MalariaGen
	S1721A	MalariaGen
	T1381A	MalariaGen
	V348I	MalariaGen
	V72I	MalariaGen
	V836I	MalariaGen

*Pf*KIC7 (PF3D7_0813000)	I130V	MalariaGen
	N434S	MalariaGen
	N596K	MalariaGen
	P318S	MalariaGen
	S576N	MalariaGen
*Pf*KIC9 (PF3D7_1442400)	D1345N	MalariaGen
	D1879V	MalariaGen
	D2018E	MalariaGen
	D388E	MalariaGen
	D734Y	MalariaGen
	E1861K	MalariaGen
	G674V	MalariaGen
	G984R	MalariaGen
	H1710R	MalariaGen
	I1280L	MalariaGen
	I1688V	MalariaGen
	I429S	MalariaGen
	I960V	MalariaGen
	K1039N	MalariaGen
	K1626N	MalariaGen
	K589R	MalariaGen
	K837I	MalariaGen
	L279I	MalariaGen
	M1507I	MalariaGen
	M618T	MalariaGen
	N1402K	MalariaGen
	N286H	MalariaGen
	N927H	MalariaGen
	P249Q	MalariaGen
	Q1045E	MalariaGen
	S1549N	MalariaGen
	S282N	MalariaGen
	S341N	MalariaGen
	S753T	MalariaGen
	T990R	MalariaGen
	T996I	MalariaGen
	V1586I	MalariaGen
	V2072M	MalariaGen
	Y293N	MalariaGen
*Pf*MyosinF (PF3D7_1329100)	C1196S	MalariaGen & Sequencing Ghana
	G873D	MalariaGen
	H1587R	Sequencing Ghana
	I2068T	MalariaGen
	L1165V	MalariaGen
	M1872L	MalariaGen & Sequencing Ghana
	N1615K	Sequencing Ghana
	V1193E	MalariaGen
	I568V*	Sequencing Ghana
	T1930S*	Sequencing Ghana
	1896S**	Sequencing Ghana
	1728S**	Sequencing Ghana
	1870S**	Sequencing Ghana

PfUBP1 (PF3D7_0104300)	D2704E	MalariaGen
	D2925N	MalariaGen
	G2810C	MalariaGen
	K3013Q	MalariaGen
	M2618V	MalariaGen
	N2165I	MalariaGen
	N2669S	MalariaGen
	Q2355E	MalariaGen
	Y2530H	MalariaGen
	N1710S*	Sequencing Ghana
	K1914N*	Sequencing Ghana
	E1915K*	Sequencing Ghana
	1283F**	Sequencing Ghana

4.5.2   *Pf*KIC1 (PF3D7_0606000)

The candidate *Pf*KIC1 (PF3D7_0606000) is a protein encoded by a gene located on chromosome 6 with a predicted protein comprising 1063 amino acids (aa). Functional analysis of the protein sequence using InterPro 84.0 yielded no domain prediction for this candidate. Seventeen different non-synonymous mutations were all simultaneously introduced into the genomic *pfkic*1 using the SLI-system, resulting in the following amino acid changes of the encoded protein compared to 3D7 sequence: N179K / K231E / S394N / S429P / D483H / D535G / V543T / N633Y / N634Y / K676N / T679A / N688Y / D862V / I873M / K883T / N971S / Q1042K (Fig. 25 A). A sequence encoding GFP was also added, to permit detection of the mutated protein and to facilitate detection of integration of the construct. PCR confirmed correct integration of the construct, resulting in a parasite line expressing the modified *Pf*KIC1 with the 17 mutations (KIC1^{mutpool}) from the endogenous locus (Fig 25 B). To exclude the possibility that the GFP-tag interfered with the result, a parasite line wherein the wild type *pfkic*1 gene was fused to the sequence encoding 2xFKBP-GFP-2xFKBP (KIC1^{wt}) (kindly provided by Jakob Birnbaum) was used as a control. KIC1^{mutpool} showed a location in foci of which one was always at the parasite's digestive vacuole, which is typical for *Pf*Kelch13 and the Kelch13 compartment members (Fig 25 C) resembling the location of KIC1^{wt} in previous work (Birnbaum, 2017). Per definition 1% marks the cut-off value for parasite resistance to artemisinin by RSA (Witkowski & Amaratunga *et al.*, 2013). Since KIC1^{mutpool} parasites reached a mean survival rate of only 0.12%, comparable to the KIC1^{wt} control, it can be assumed that none of the mutations changed the susceptibility of the parasites to ART (Fig 25 D).
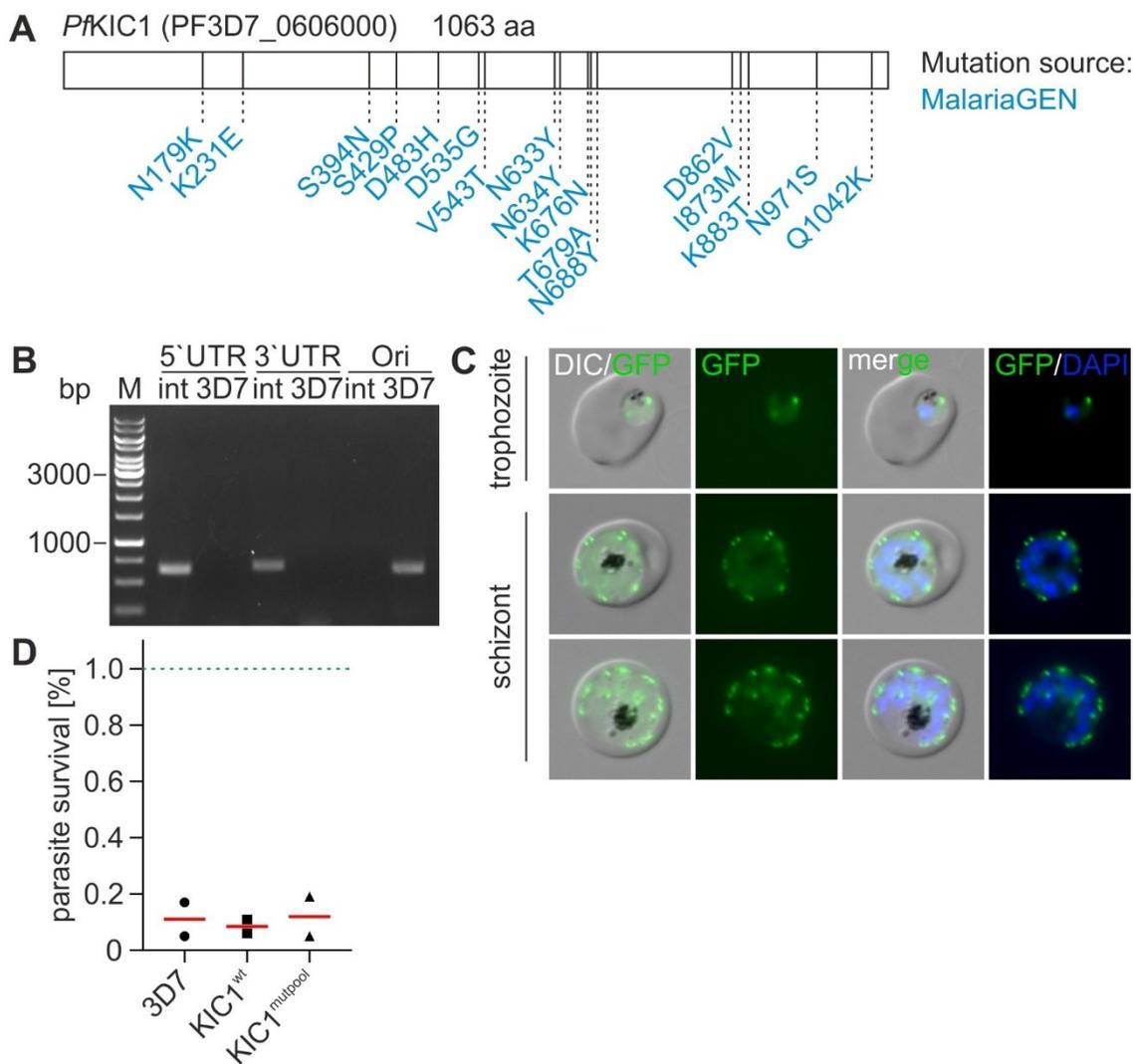
Figure 25) Analysis of mutant *Pf*KIC1 (PF3D7_0606000) A) Scheme showing mutations included in KIC1mutpool. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain KIC1mutpool parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 632 bp) and 3`-integration (3`UTR, 678 bp) and to demonstrate absence of original locus in the KIC1mutpool parasites (Ori, 638 bp). 3D7 gDNA was used as reference (ori 3D7 638 bp). **C)** Live cell images of different developmental stages of knock-in cell line KIC1mutpool. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.5.3 *Pf*KIC2 (PF3D7_1227700)

The candidate *Pf*KIC2 (PF3D7_1227700) is located on chromosome 12 and encodes a protein of 771 aa. InterPro 84.0 predicted no domain for *Pf*KIC2. The SLI-system was used to simultaneously incorporate seven non-synonymous SNPs chosen from MalariaGEN into the genomic *pfkic2* locus, resulting in the amino acid changes G167D / E207Q / K321N / E343K / N449T / Y472F / K651M compared to 3D7 sequence (Fig 26 A). In addition, the sequence encoding GFP was C-terminally added to the mutated *Pf*KIC2. Correct integration of the gene modification construct was confirmed by PCR (Fig. 26 B). The resulting KIC2^{mutpool} showed a comparable localization in a pattern typical of *Pf*Kelch13 and its compartment members by fluorescence microscopy (Fig. 26 C) compared to a previously generated endogenously FKBP and GFP-tagged unmodified *Pf*KIC2 (KIC2^{wt}) (kindly provided by Jakob Birnbaum). *In vitro* DHA resistance of KIC2^{mutpool} parasites was tested in a standard RSA. The KIC2^{mutpool} parasites showed a mean survival rate of 0.05% and showed no significant change in sensitivity to DHA compared to the KIC2^{wt} cell line that reached a mean survival rate of 0.08% (Fig 26 D). Since the survival rates of the KIC2^{mutpool} parasites was below the 1% cut-off value (Witkowski & Amaratunga *et al.*, 2013), this indicated that none of the seven mutations confers resistance to ART.
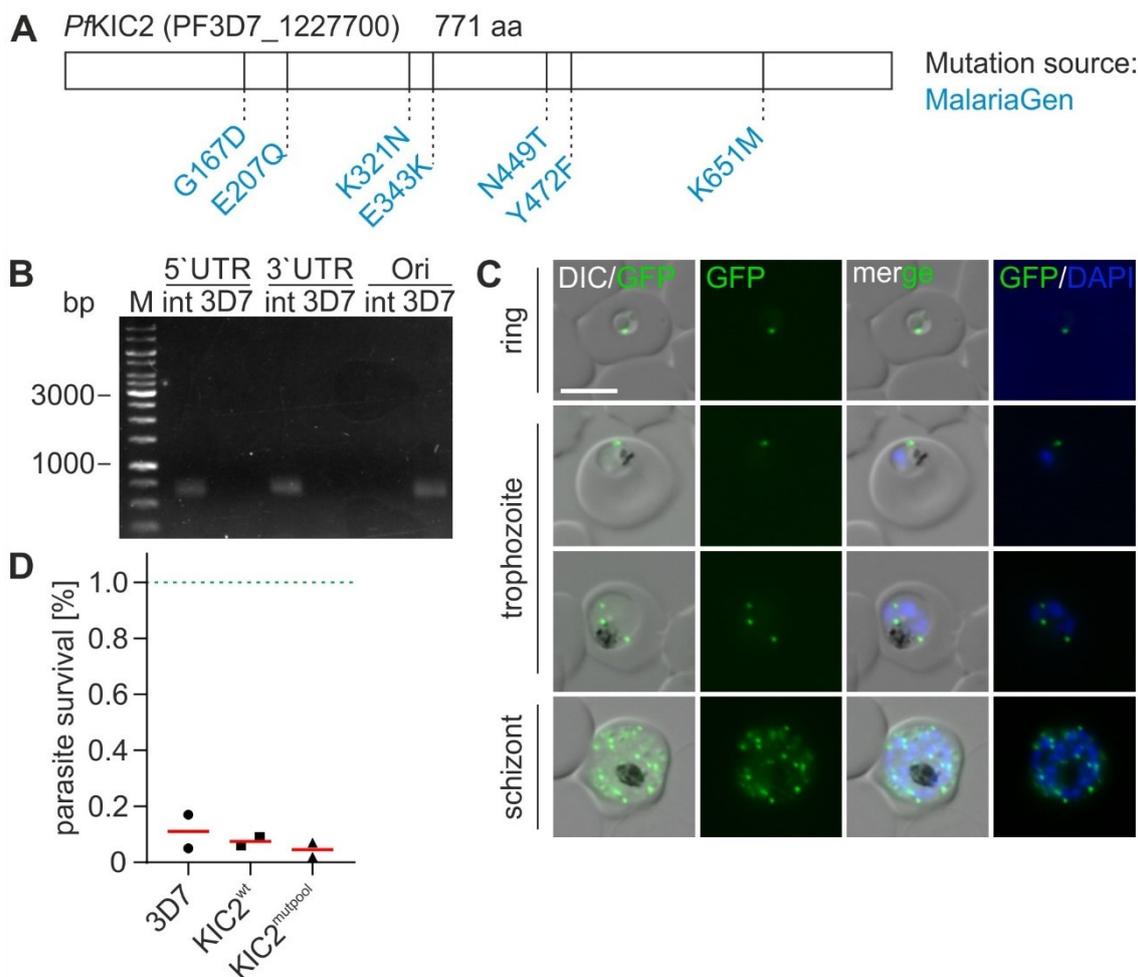
Figure 26) Analysis of mutant *Pf*KIC2 (PF3D7_1227700) A) Scheme showing mutations included in KIC2^mutpool. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain KIC2^mutpool parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 627 bp) and 3`-integration (3`UTR, 652 bp) and to demonstrate absence of original locus in the KIC2^mutpool parasites (Ori, 613 bp). 3D7 gDNA was used as reference (ori 3D7 613 bp). **C)** Live cell images of different developmental stages of knock-in cell line KIC2^mutpool. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler^TM 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.5.4   *Pf*KIC4 (PF3D7_1246300)

Kelch13 interacting candidate 4 (PF3D7_1246300) is a protein of 888 aa and the encoding gene is located on chromosome 12. According to InterPro 84.0, the C-terminal part of this protein contains a clathrin adaptor domain. In total, 12 non-synonymous SNPs were chosen

to be included in the mutation pool, leading to the following amino acid changes in *Pf*KIC4 compared to 3D7 sequence: D120N / I121V / N123S / P170S / Y179C / M375V / N405S / V619A / P621A / N624I / N661H / T888A (Fig. 27 A). The SLI system was used to integrate the construct harboring the 12 mutations into the 3D7 genome resulting in a parasite line expressing the modified *Pf*KIC4 (KIC4^{mutpool}) fused to GFP from the endogenous locus and correct integrations was confirmed by PCR (Fig 27 B). KIC4^{mutpool} was expressed in all asexual developmental stages and localized in foci, similar to wild type *Pf*KIC4 fused to FKBP and GFP (KIC4^{wt}) in a pattern typical for *Pf*Kelch13 and its compartment proteins (Birnbaum, 2017) (Fig 27 C). The resistance of KIC4^{mutpool} parasites to artemisinin was determined by standard RSA and showed no change of responsiveness to the drug compared to 3D7 or the KIC4^{wt} control parasites (Fig. 27 D). The mean survival rate of KIC4^{mutpool} was 0.08%, whereas the mean survival rate of KIC4^{wt} was 0.65%, both clearly below the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013). Hence, none of the SNPs inserted into *Pf*KIC4 influences the responsiveness of the parasite to ART.

Figure 27) Analysis of mutant *Pf*KIC4 (PF3D7_1246300) A) Scheme showing mutations included in KIC4^{mutpool}. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain KIC4^{mutpool} parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 628 bp) and 3`-integration (3`UTR, 671 bp) and to demonstrate absence of original locus in the KIC4^{mutpool} parasites (Ori, 627 bp). 3D7 gDNA was used as reference (ori 3D7 627 bp). **C)** Live cell images of different developmental stages of knock-in cell line KIC4^{mutpool}. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.5.5   *Pf*KIC5 (PF3D7_1138700)

Kelch13 interaction candidate 5 (PF3D7_1138700) is encoded on chromosome 11 and is a protein of 1870 aa. The C-terminal part of this protein from position 1849 to 1866 contains a hydrophobic region predicted to be a transmembrane domain according to InterPro 84.0, but not TMHMM which is typically used to predict such domains in *P. falciparum* proteins (VEuPathDB, 2021). Overall, 33 non-synonymous SNPs were included in the mutation pool resulting in the following amino acid changes compared to 3D7 sequence: V72I / H138Y / D169N / M324V / E497Q / P516S / N525S / F570L / G727E / K751Q / V836I / M975I / K985N / R1040K / E1055G / Q1127K / H1198Y / N1268H / T1381A / Q1421K / E1646G / S1721A / Q110E / K258R / G264D / V348I / K384I / A471T / S1044R / L1066P / E1354D / K1411R / G1417D (Fig 28 A). A C-terminal GFP tag was also added. After successful selection of the cell line expressing the modified *Pf*KIC5 from the endogenous *pfkic5* locus (KIC5^{mutpool}), correct integration was confirmed by PCR (Fig. 28 B). The localization of KIC5^{mutpool} was assessed by live cell imaging (Fig 28 C). KIC5^{mutpool} was expressed throughout the asexual cycle and the localization was typical for a *Pf*Kelch13 compartment protein and was similar to the localization of the unmodified *Pf*KIC5 fused to 2xFKBP-GFP-2xFKBP (KIC5^{wt}) that had previously been generated (kindly provided by Jakob Birnbaum) and was used here as a control for the RSA. The KIC5^{mutpool} parasites reached a mean survival rate of 0.15% in a standard RSA, while the KIC5^{wt} parasites showed a mean survival rate of 0.3% (Fig 28 D). Since both values were comparable and below the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013), it can be concluded that the 33 mutations introduced into *Pf*KIC5 in the KIC5^{mutpool} parasites did not render them resistant to ART.
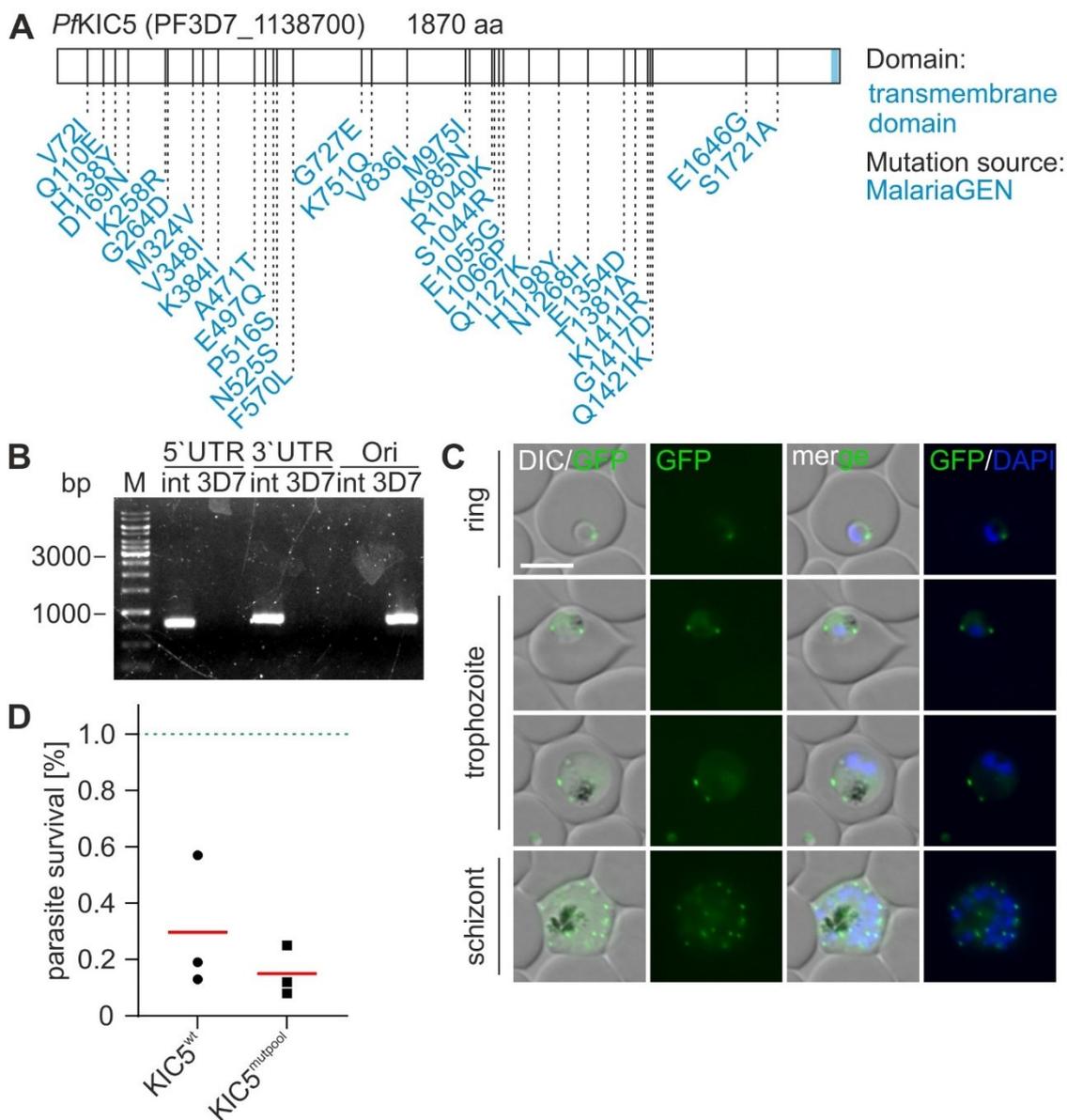
Figure 28) Analysis of mutant *Pf*KIC5 (PF3D7_1138700) A) Scheme showing mutations included in KIC5^mutpool. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain KIC5^mutpool parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 779 bp) and 3`-integration (3`UTR, 848 bp) and to demonstrate absence of original locus in the KIC5^mutpool parasites (Ori, 817 bp). 3D7 gDNA was used as reference (ori 3D7 817 bp). **C)** Live cell images of different developmental stages of knock-in cell line KIC5^mutpool. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.5.6 *Pf*KIC7 (PF3D7_0813000)

The gene of the Kelch13 interacting candidate *Pf*KIC7 (PF3D7_0813000) is located on chromosome 8 and encodes a protein of 599 aa. Search on InterPro 84.0 predicted no protein domains for this candidate. Using the SLI-system, five different non-synonymous mutations were simultaneously introduced into the genomic *pfkic7,* leading to the following amino acid changes compared to 3D7 sequence: I130V / P318S / N434S / S576N / N596K (Fig 29 A). Correct integration of the construct was confirmed by PCR (Fig. 29 B). The resulting parasite line expressing a modified *Pf*KIC7 (KIC7^{mutpool}) from the endogenous locus showed that KIC7^{mutpool} was expressed throughout the asexual life cycle in foci showing the typical pattern of *Pf*Kelch13 compartment proteins and similar to the wild type *Pf*KIC7 fused to GFP and FKBP (KIC7^{wt}) (Birnbaum, 2017) (Fig 29 C). Parasite survival rate of the KIC7^{mutpool} parasites was tested by standard RSA and compared to the survival of KIC7^{wt}. The mean survival rate of KIC7^{mutpool} was 0.13%, whereas the KIC7^{wt} parasites showed a survival rate of 0.26% (Fig 29 D). Since both parasite lines showed comparable survival rates and were clearly below the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013), it can be assumed that none of the selected non-synonymous mutations rendered the parasites resistant to ART.
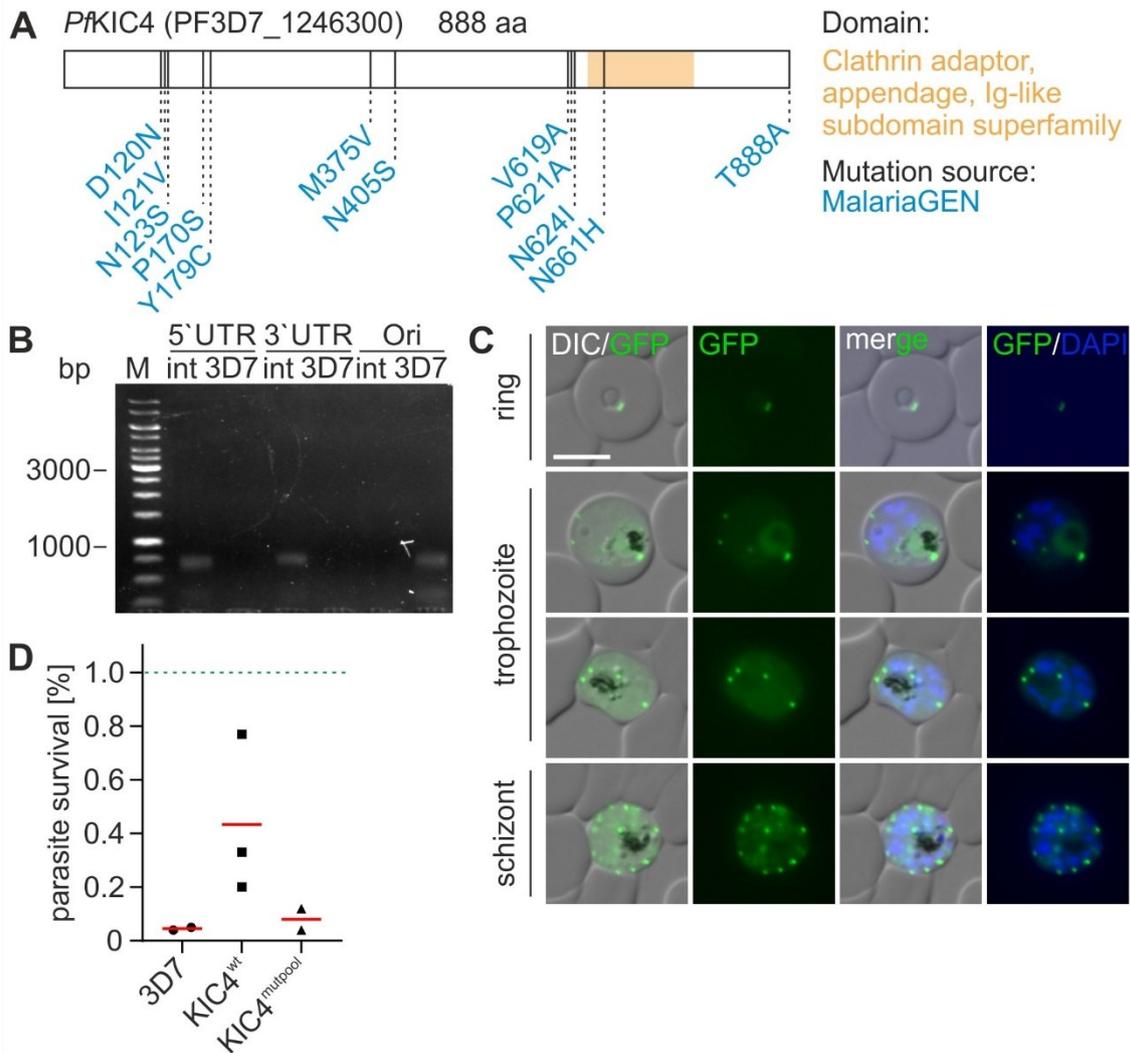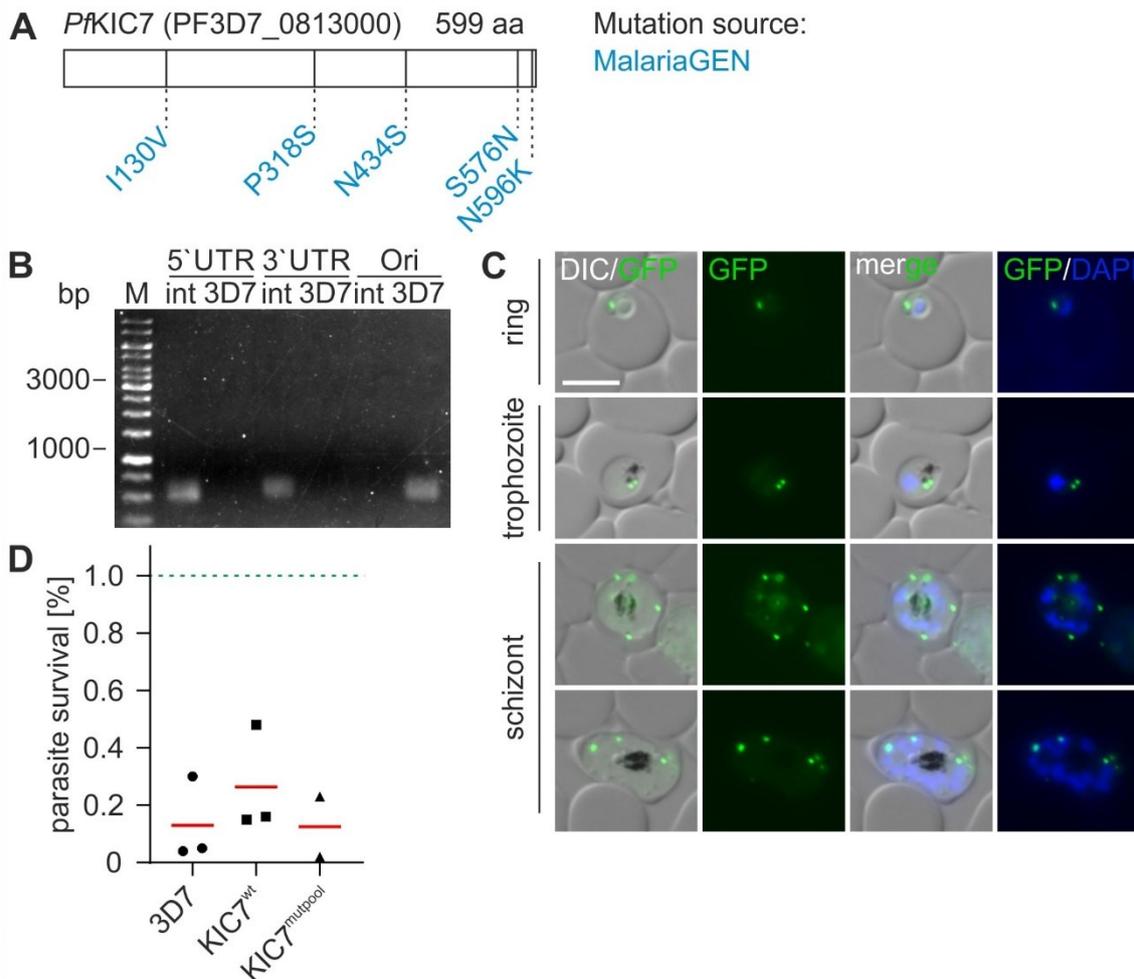
Figure 29) Analysis of mutant *Pf*KIC7 (PF3D7_0813000) A) Scheme showing mutations included in KIC7mutpool. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain KIC7mutpool parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 485 bp) and 3`-integration (3`UTR, 522 bp) and to demonstrate absence of original locus in the KIC7mutpool parasites (Ori, 483 bp). 3D7 gDNA was used as reference (ori 3D7 483 bp). **C)** Live cell images of different developmental stages of knock-in cell line KIC7mutpool. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.5.7 *Pf*KIC9 (PF3D7_1442400)

The candidate *Pf*KIC9 (PF3D7_1442400) is a protein encoded by a gene located on chromosome 14 encoding a protein of 2113 aa. According to InterPro, no protein domain is predicted for this candidate. Overall, 34 non-synonymous mutations were introduced into

115

the genomic *pfkic9* locus using the SLI-system resulting in the following amino acid changes compared to 3D7 sequence: L279I / N286H / S341N / I429S / M618T / G674V / K837I / N927H / I960V / T990R / K1039N / Q1045E / I1280L / D1345N / M1507I / V1586I / K1626N / I1688V / H1710R / E1861K / D1879V / P249Q / S282N / Y293N / D388E / K589R / D734Y / S753T / G984R / T996I / N1402K / S1549N / D2018E / V2072M (Fig. 30 A). After successful transfection of the plasmid and selection of the integrants, correct integration was confirmed by PCR (Fig. 30 B), resulting in a cell line expressing a modified version of *Pf*KIC9 (KIC9^{mutpool}). Fluorescence microscopy showed that the localization of KIC9^{mutpool} was typical for a Kelch13 compartment protein (Fig. 30 C) and comparable to the control cell line expressing the wild type *Pf*KIC9 fused to GFP and FKBP (KIC9^{wt}) (kindly provided by Jakob Birnbaum). KIC9^{mutpool} was expressed over the entire asexual developmental cycle with a very faint signal in ring stages and the typical foci pattern in later stages (Fig 30 C). In the standard RSA, KIC9^{mutpool} parasites had a mean survival rate of 0.16%, whereas KIC9^{wt} showed a mean survival rate of 0.09% (Fig 30 D). These survival rates were comparable to that of 3D7 (mean survival rate of 0.21%) and lower than the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013). This indicates that none of the SNPs introduced into *Pfkic9* rendered the parasites ART resistant.
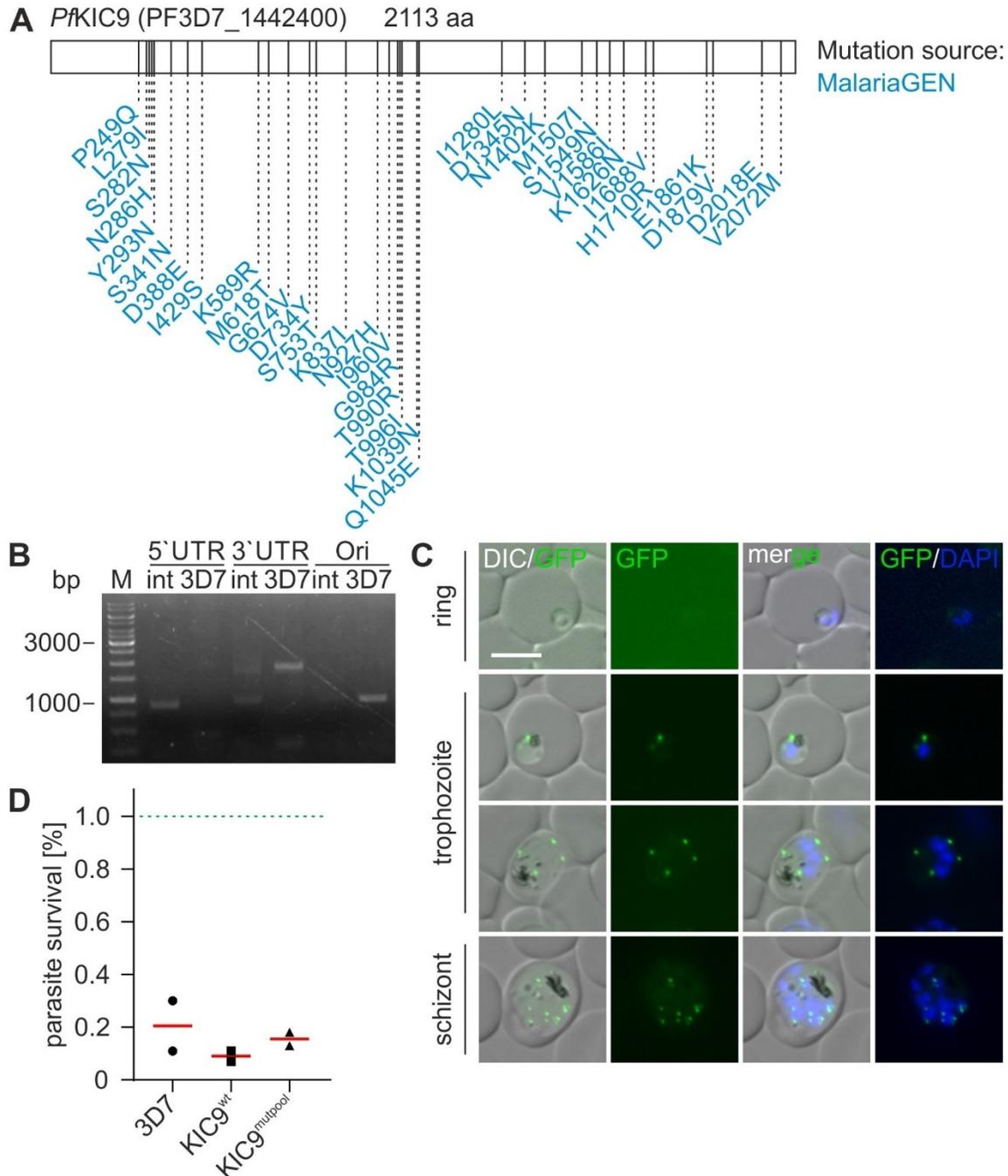
Figure 30) Analysis of mutant *Pf*KIC9 (PF3D7_1442400) A) Scheme showing mutations included in KIC9^mutpool. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain KIC9^mutpool parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 871 bp) and 3`-integration (3`UTR, 921 bp) and to demonstrate absence of original locus in the KIC9^mutpool parasites (Ori, 914 bp). 3D7 gDNA was used as reference (ori 3D7 914 bp). **C)** Live cell images of different developmental stages of knock-in cell line KIC9^mutpool. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler^TM 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.5.8   *Pf*MyosinF (PF3D7_1329100)

*Pf*MyosinF (PF3D7_1329100) is a protein of 2160 aa encoded by a gene located on chromosome 13. InterPro 84.0 detected a myosin head domain in the N-terminal part in position 67 to 831. This domain is followed by two P-loop containing nucleoside triphosphate hydrolases and an IQ motif EF-hand binding site at position 1008 to 1060. In the C-terminal part, several WD40 repeats are located (Fig. 31 A). In total, eight non-synonymous mutations were included in the mutation pool, causing the following amino acid changes compared to 3D7 sequence: G873D / V1193E / C1196S / M1872L / L1165V / I2068T / H1587R / N1615K (Fig 31 A). Via the SLI system, the eight non-synonymous mutations were introduced into the endogenous *pfmyosinF* locus resulting in a cell line with the modified *Pf*MyosinF (MyosinF^mutpool^) as verified by PCR (Fig. 31 B). Localization of MyosinF^mutpool^ was assessed by live cell imaging. The protein was found to be mainly expressed in trophozoite to schizont stages but also a very faint signal was visible in ring stages (see section 4.2.1) (Fig 31 C). When MyosinF^mutpool^ parasites were tested in standard RSA, a mean survival rate of 0.11% was observed, which was below the 1% cut-off value (Witkowski & Amaratunga *et al.*, 2013), indicating that the selected mutations did not render parasites resistant to ART (Fig 31 D).
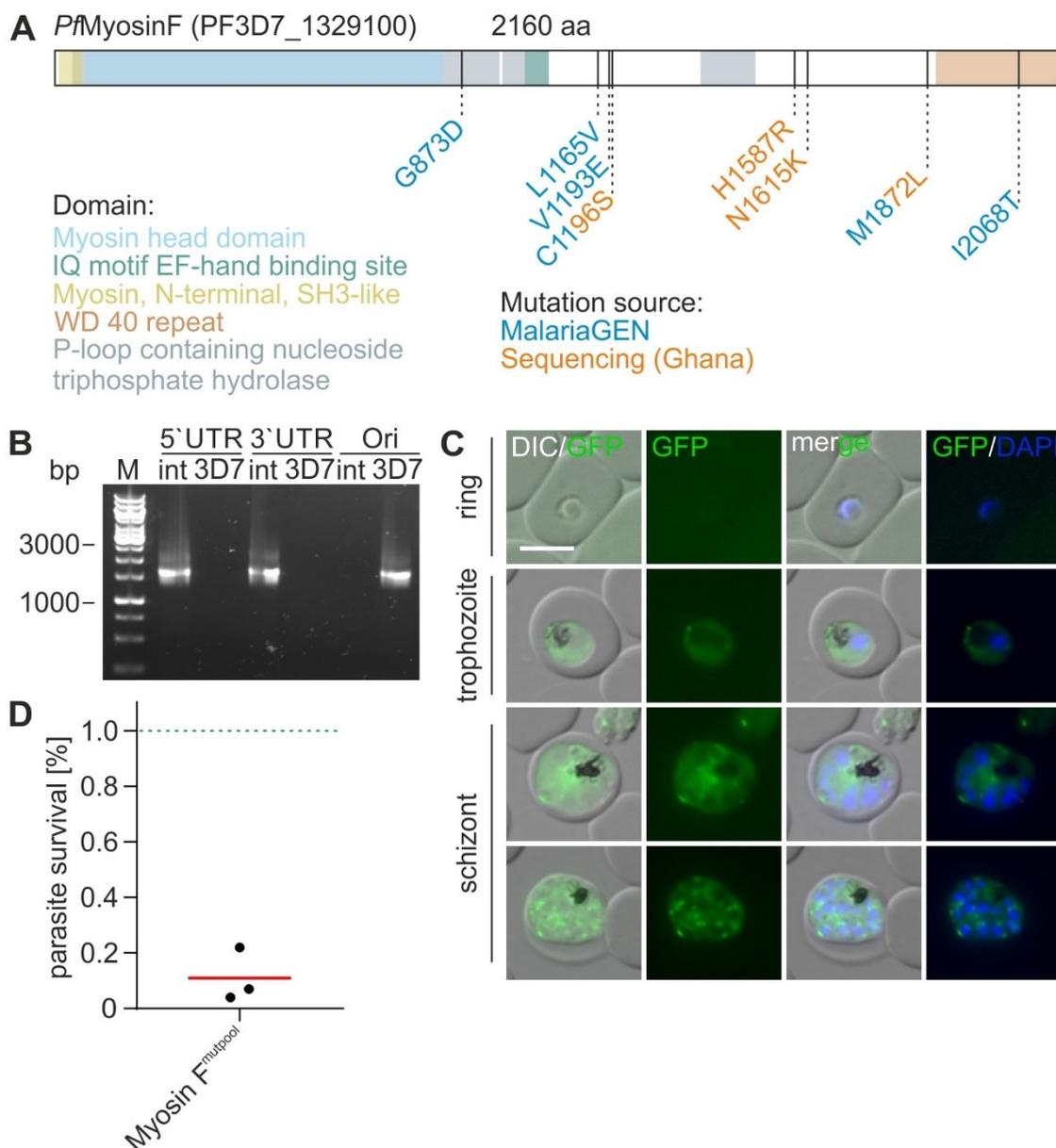
Figure 31) Analysis of mutant *Pf*MyosinF (PF3D7_1329100) A) Scheme showing mutations included in MyosinF[mutpool]. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain MyosinF[mutpool] parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 1639 bp) and 3`-integration (3`UTR, 1676 bp) and to demonstrate absence of original locus in the MyosinF[mutpool] parasites (Ori, 1630 bp). 3D7 gDNA was used as reference (ori 3D7 1630 bp). **C)** Live cell images of different developmental stages of knock-in cell line MyosinF[mutpool]. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

119

4.5.9 *Pf*UBP1 (PF3D7_0104300)

A further member of the Kelch13 compartment is the ubiquitin carboxyl-terminal hydrolase, *Pf*UBP1 (PF3D7_0104300) that is encoded by a gene located on chromosome 1 that encodes a protein of 3499 aa. Domain prediction via InterPro 84.0 revealed a ubiquitin specific protease domain from position 3170 to 3489. Using the SLI-system, nine mutations were simultaneously incorporated into the endogenous *pfubp1* locus that resulted in the following amino acid changes compared to 3D7 sequence: N2165I / Q2355E / Y2530H / M2618V / N2669S / D2704E / G2810C / D2925N / K3031Q (Fig 32 A). PCR verified correct integration of the construct, resulting in a cell line expressing a mutated *Pf*UBP1 (UBP1^{mutpool}) from endogenous locus (Fig 32 B). The protein carrying the nine mutations showed a similar localization to *Pf*UBP1 2xFKBP-GFP of the wild type parasite line (UBP1^{wt}) (Birnbaum, 2017) (Fig 32 C). In the standard RSA, the 1% cut-off value (Witkowski & Amaratunga *et al.*, 2013) was not exceeded, neither by UBP1^{mutpool} (mean survival rate 0.15%), nor by UBP1^{wt} (mean survival rate 0.13%), suggesting that none of the introduced mutations change the responsiveness of the parasites to ART (Fig 32 D).
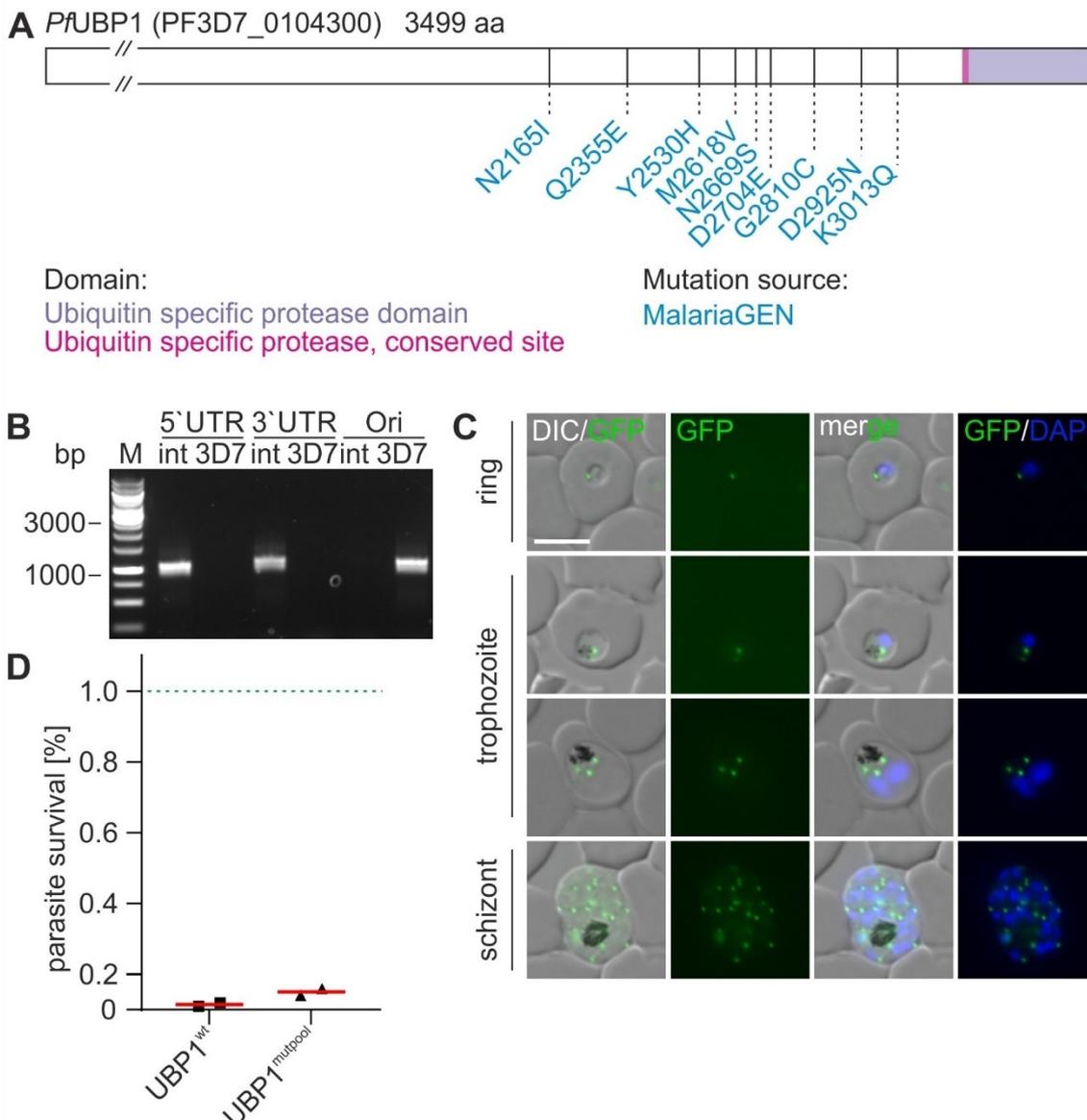
Figure 32) Analysis of mutant *Pf*UBP1 (PF3D7_0104300) A) Scheme showing mutations included in UBP1^mutpool. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain UBP1^mutpool parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 1092 bp) and 3`-integration (3`UTR, 1179 bp) and to demonstrate absence of original locus in the UBP1^mutpool parasites (Ori, 1129 bp). 3D7 gDNA was used as reference (ori 3D7 1129 bp). **C)** Live cell images of different developmental stages of knock-in cell line UBP1^mutpool. **D)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *DIC*, differential interference contrast; *GFP*, green fluorescent protein; *DAPI*, 4´,6-Diamidin-2-phenylindol; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *bp*, base pairs; *int*, integrant; *ori*; original locus; *wt*, wildtype; *mut*, mutant.

4.6 Non-synonymous SNPs tested individually on their contribution to ART resistance

4.6.1 Non-synonymous SNPs in *Pf*MyosinF (PF3D7_1329100) do not influence ART resistance

*Pf*MyosinF is part of the Kelch13 compartment and partially co-localizes with *Pf*Kelch13 (see section 4.2.1). Besides testing various non-synonymous SNPs in the multi-pooled screening (section 4.5.8), five different non-synonymous SNPs were tested individually for their capability to induce ART resistance. The first reason for not including these SNPs into the pool was because four of the five SNPs are located in the N-terminal region of *pfmyosinF* which would have complicated their inclusion due to the large size of this gene. Secondly, some of these SNPs were selected for testing after the plasmid for the multipool analysis was already prepared for transfection. In total, five SLI plasmids were generated, each intended to result in one of the following amino acid changes in *Pf*MyosinF compared to 3D7: N277S / D500G / Q635H / S969P / S1457L (Fig 33 A). Integration into the endogenous *pfmyosinF* locus was successful for all but the plasmid generating the amino acid change N277S, which failed to correctly integrate in 12 independent integration attempts. Correct integration of the remaining constructs was confirmed by PCR (see Appendix F.1), resulting in the four different parasite lines MyoF^{D500G}, MyoF^{Q635H}, MyoF^{S969P} and MyoF^{S1457L}. Standard RSA was performed with each of the cell lines and showed a mean parasite survival rate of 0.08% for MyoF^{D500G}, 0.03% for MyoF^{Q635H}, 0.07% for MyoF^{S969P} and 0.05% for MyoF^{S1457L} (Fig 33 B). All survival rates were therefore below the cut-off value of 1% that defines resistance (Witkowski & Amaratunga *et al.*, 2013), indicating that none of the tested non-synonymous SNPs rendered parasites resistant to ART.
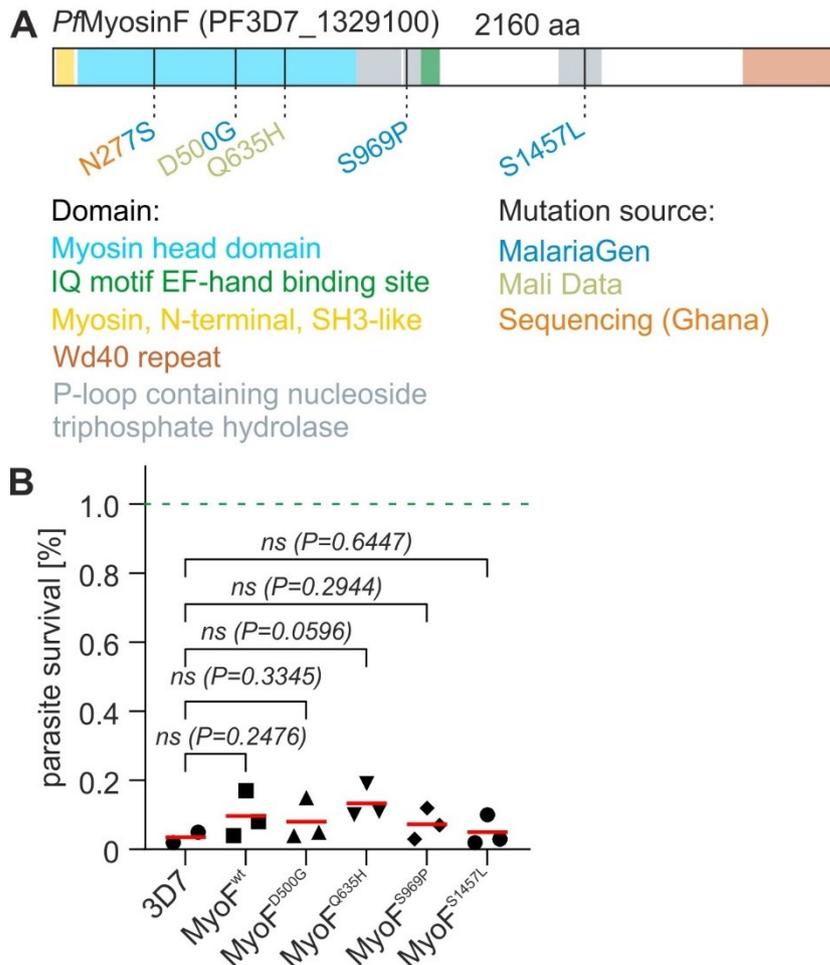
**Figure 33) Individually tested SNPs of *pfmyosinF*. A)** Scheme showing non-synonymous SNPs of *Pf*MyosinF chosen for separate testing in standard RSA. SNPs shown in more than one color were found in multiple sources. **B)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch´s t test. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *wt*, wild type.

4.6.2   Parasites with a SNP in *Pf*AP-2α (PF3D7_0617100) show a very mild reduction in susceptibility to ART compared to 3D7

The protein *Pf*AP-2 α comprises 1236 aa and is encoded by a gene located on chromosome 6. This candidate is interesting in the context of ART resistance since it is part of the AP-2 adaptor complex that is enrolled in endocytosis and it was shown before that other components of the AP-2 adaptor complex co-localize to *Pf*Kelch13 and influence ART resistance (see section 4.4.2). In 2018, *Rocamora et al.* reported that 3D7 parasites

artificially selected for ART contained a non-synonymous SNP leading to an H817P change in *PfAP-2α* (Rocamora *et al.*, 2018) (Fig 34 A). To test whether this non-synonymous SNP renders parasites resistant to ART, the SLI-system was used to incorporate the SNP into the endogenous *pfap-2α* locus of 3D7 parasites (AP-2α^{H817P} cell line, kindly provided by Jakob Birnbaum), and a standard RSA was performed. In the standard RSA, the mean survival rate of the AP-2α^{H817P} parasites was 0.68% which is under the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013) (Fig 34 B). While this indicated that this SNP did not render the parasites resistant to ART according to the current 1% cut off, there was nevertheless a significant difference to the mean survival rate of the 3D7 control (mean survival rate 0.15%), indicating that this codon change resulted in a very mild increase in ART resistance.
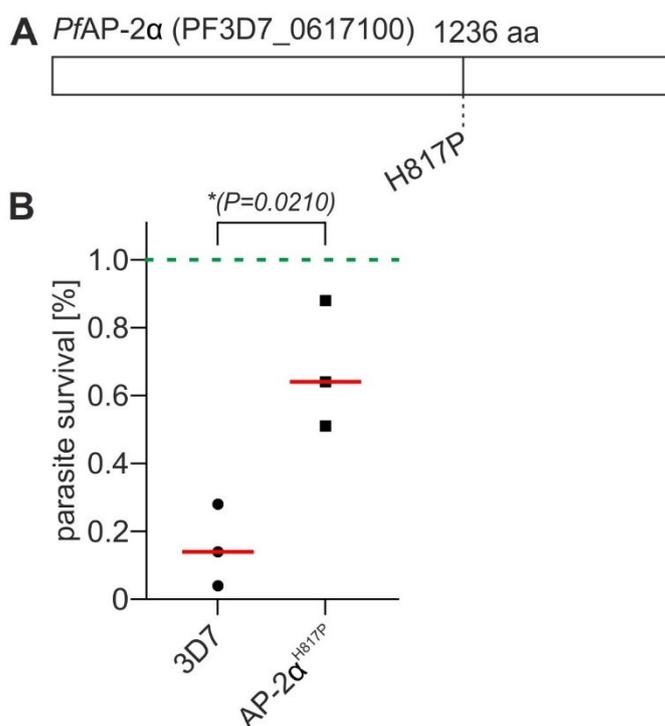
Figure 34) Individual tested SNP of *pfap-2α*. A) Scheme showing non-synonymous SNP of *Pf*AP-2α chosen for separate testing in standard RSA. **B)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch´s t test. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid.

4.6.3 Asian non-synonymous SNP identified in *Pf*Eps15 (PF3D7_1025000) does not influence ART resistance

A genomic surveillance study including whole-genome sequencing data of *P. falciparum* isolates collected in Thailand from 2001 and 2014 reported a series of SNPs associated with ART resistance which included a non-synonymous SNP leading to the amino acid change K447R in *Pf*Eps15 (Cerqueira *et al.*, 2017) (Fig 35 A). As *Pf*Eps15 is part of the Kelch13 compartment and its inactivation renders parasites resistant to ART (see section 4.4.2), it was here tested if the K447R change in this protein decreases the sensitivity of the parasite to ART. In order to test this, the SLI-system was used to introduce the corresponding mutation into the *pfeps15* locus. At the same time, the sequence encoding *gfp* was added resulting in the cell line Eps15^{K447R} (kindly provided by Jakob Birnbaum). When a standard RSA was performed with the Eps15^{K447R} cell line, parasites reached a mean survival rate of 0.17% which is under the cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013), indicating that the parasites with the modified *pfeps15* locus did not become resistant to ART (Fig 35 B).
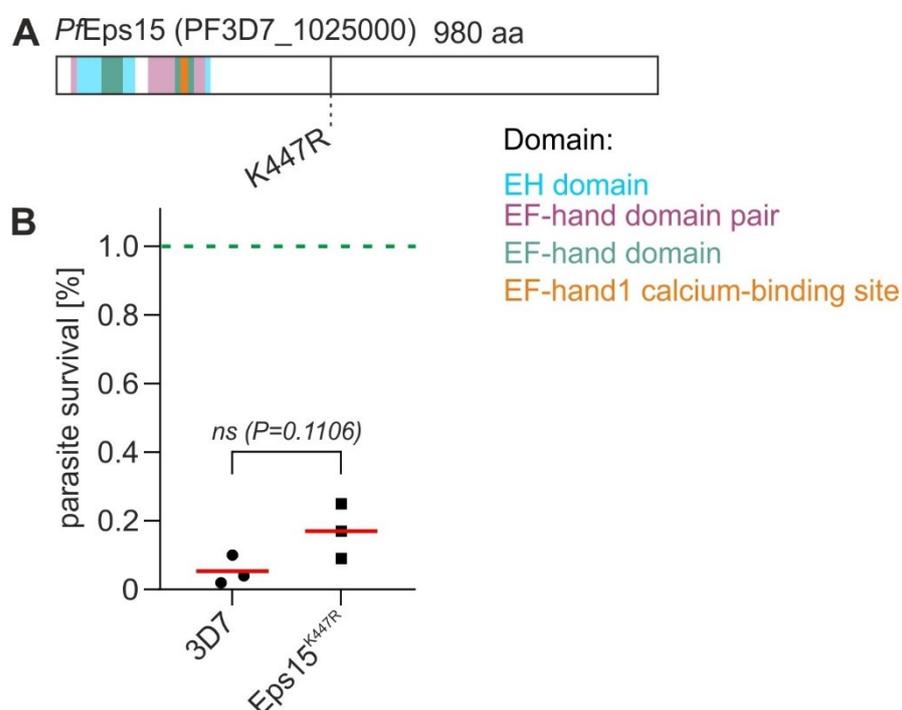
Figure 35) Individual tested SNP of *pfeps15*. A) Scheme showing non-synonymous SNP of *Pf*Eps15 chosen for separate testing in standard RSA. **B)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch´s t test. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid.

4.6.4 *Pf*UBP1 (PF3D7_0104300) SNP detected in Asia confers tolerance to ART

The candidate *Pf*UBP1 belongs to the Kelch13 compartment (Birnbaum *et al.*, 2020) and influences sensitivity of the parasite to ART when the protein is inactivated (see section 4.4.2). Genome surveillance data of field isolates collected in Thailand indicated that a non-synonymous SNP at codon 3138 of *pfubp1,* resulting in the amino acid change R3138H, was associated with ART resistance ((Cerqueira *et al.*, 2017) Fig 36 A). To test whether this mutation indeed leads to ART resistant parasites, the SLI system was used to incorporate this SNP into the endogenous *pfubp1* locus. In addition, the mutated protein was tagged with a sequence encoding a 3xHA tag. Correct integration of the construct was confirmed by PCR (Fig 36 B), resulting in the cell line UBP1^{R3138H} and expression of the modified protein was verified using western blot analysis (expected molecular weight 420 kDa) (Fig 36 C). In the western blot a second band at ~460 kDa was identified which corresponds with the size of the unskipped protein. Fluorescence microscopy was performed with formaldehyde/glutaraldehyde-fixed UBP1^{R3138H} parasites which showed an expression of the protein throughout the asexual replication in the RBC (Fig 36 D). In trophozoite and schizont stages, always one focus was located next to the food vacuole, similar to the other members of the Kelch13 compartment. The UBP1^{R3138H} cell line was tested in a standard RSA. These experiments showed a mean survival rate of 1.51% with the UBP1^{R3138H} parasites which was significantly different from 3D7 parasites (mean survival rate 0.23%) and the control cell line, a similarly 3xHA-tagged UBP1^{wt} (mean survival rate 0.13%) (Fig 36 E). Since the survival of the UBP1^{R3138H} parasites was higher than that resistance defining cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013), it can be concluded that the R3138H mutation renders the parasite resistant to ART.
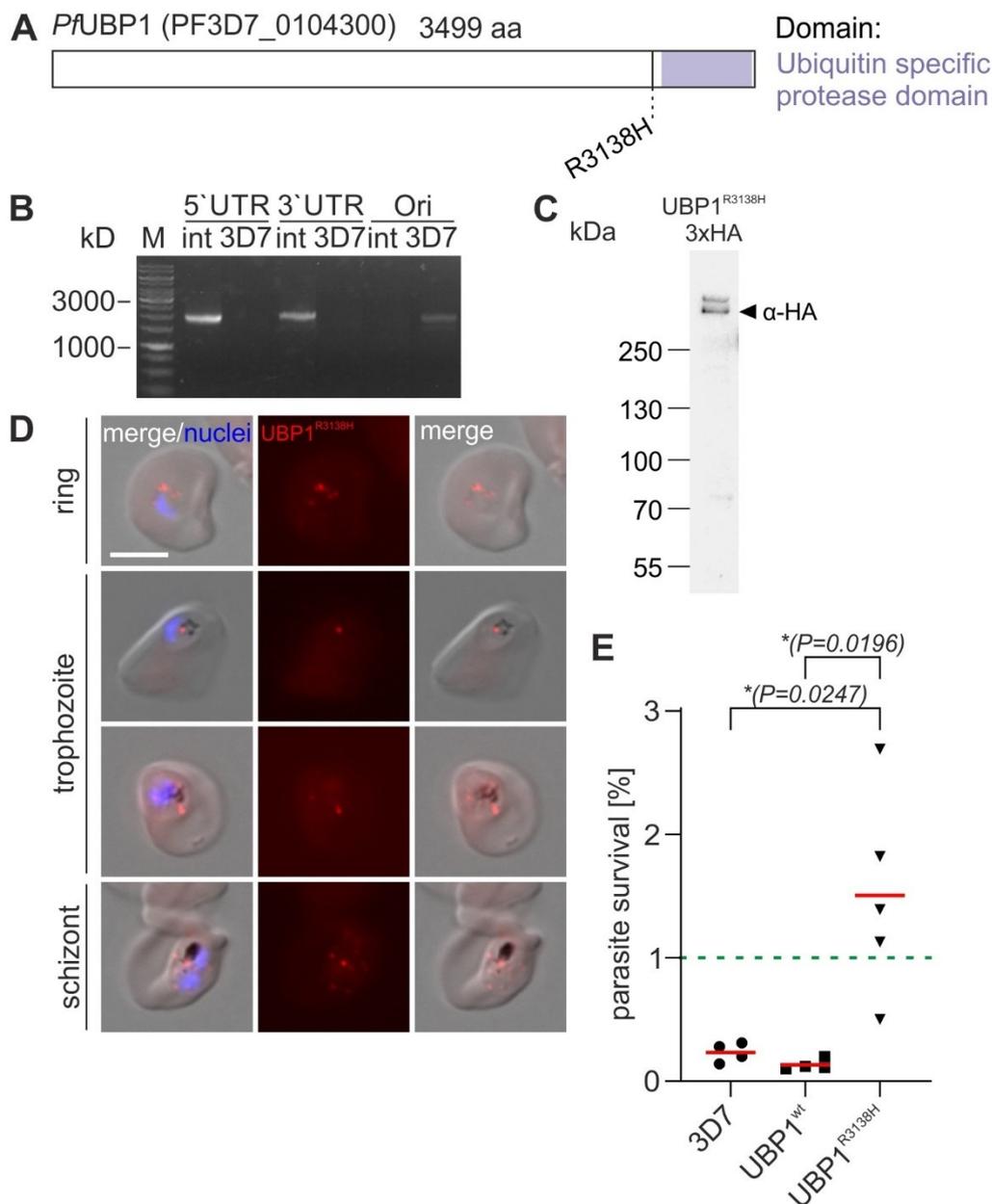
Figure 36) UBP1^{R3138H} renders parasite resistant to ART. A) Scheme showing non-synonymous SNP of *Pf*UBP1 chosen for separate testing in standard RSA. **B)** Agarose gel showing PCR products demonstrating correct integration of the SLI plasmid into 3D7 genome to obtain UBP1^{R3138H} parasites. Primers (see Appendix C) were used to confirm 5`-integration (5`UTR, 2103 bp) and 3`-integration (3`UTR, 2150 bp) and to demonstrate absence of original locus in the UBP1^{R3138H} parasites (Ori, 2120 bp). 3D7 gDNA was used as reference (ori 3D7 2120 bp). **C)** Western blot analysis with protein extracts of saponin-lysed parasites of UBP1^{R3138H} parasites. α-HA (from rat) was used to detect UBP1^{R3138H} (expected MW of 420 kDa). Molecular weight standard is indicated in kDa. Full blots are shown in Appendix F.2. **D)** IFA images of formaldehyde/glutaraldehyde-fixed UBP1^{R3138H} parasites. Nuclei were stained with DAPI. **E)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch´s t test. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *kD*, kilodalton; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *int*, integrant; *ori*; original locus; *HA*, hemagglutinin; *wt*, wildtype; size bar, 5μm.

4.6.5 Two non-synonymous SNPs detected in *Pf*Kelch13 (PF3D7_1343700) reduce ART susceptibility in *P. falciparum*

The gene encoding *Pf*Kelch13 is located on chromosome 13 and is translated into a protein of 726 aa. The protein consists of different domains, the N-terminal *Plasmodium* specific region, followed by the BTB/POZ domain in the middle and a C-terminal 6-blade Kelch propeller domain. In 2014, *Ariey et al* showed that single point mutations in *Pf*Kelch13 are associated with ART resistance (Ariey *et al.*, 2014), and since then various studies were performed to identify non-synonymous SNPs in *kelch13* that cause resistance to ART. Here, four non-synonymous SNPs were tested for their capability to render parasite resistant, resulting in the following amino acid changes in comparison to the 3D7 reference sequence: V520A / V520I / V589I / E612K (Fig 37 A). The SNP V520A was found 2006 in Africa during a molecular epidemiologic study and showed the highest prevalence of 4% in the Democratic Republic of Congo (Taylor *et al.*, 2015). Another SNP also located at the same codon results in the amino acid change V520I compared to 3D7 reference sequence: This SNP was identified 2014 in Asia and had a prevalence of 0.57% (Ménard *et al.*, 2016). In 2011, the codon change V589I was found in Mali with a prevalence of 1.15% (Ouattara *et al.*, 2015). The final non-synonymous SNP that was included here was E612K which was found in 2016 in Cameroon with a prevalence of 0.83% (Eboumbou Moukoko *et al.*, 2019).

Four SLI plasmids were generated to insert each of the four codon changes into the 3D7 genome and the mutated *Pf*Kelch13 gene was additionally tagged with a sequence encoding GFP and 2xFKBP. After selection of integrants, correct integration of the different constructs was confirmed by PCR (Fig 37 B, representative images of two cell lines are shown) and expression of the mutated and tagged *Pf*Kelch13 protein was confirmed by western blot analysis (Fig 37 C, representative images of two cell lines are shown), resulting in the four cell lines K13^{V520A}, K13^{V520I}, K13^{V589I}, K13^{E612K}. The localization of the modified *Pf*Kelch13 was assessed by live cell imaging and compared to a previously established wild type *Pf*Kelch13 cell line where the native protein is tagged with GFP and 2xFKBP (K13^{wt}, kindly provided by Jakob Birnbaum). Comparison of the cell lines K13^{V520A}, K13^{V520I}, K13^{V589I}, K13^{E612K} to K13^{wt} revealed no apparent difference in the localization of the mutated *Pf*Kelch13 which were found in foci in a pattern typical for K13^{wt} (Fig 37 D, representative images of two cell lines are shown). When the four parasite lines were tested in standard RSA, the K13^{E612K} parasite line showed a mean survival rate of 0.60%, and the K13^{V520I} line a mean survival rate of 0.54% (Fig 37 E). Both values were below the resistance defining cut-off value of 1% (Witkowski & Amaratunga *et al.*, 2013) and not significantly different than the survival of the K13^{wt} parasites, indicating that the two SNPs did not render the

parasite resistant to ART. In contrast, RSA with the K13^{V520A} cell line resulted in a mean parasite survival rate of 1.10%, and the K13^{V589I} parasites showed a mean survival rate of 1.92%. As these values were above the 1% cut-off for resistance although there was no significant difference compared to K13^{wt} control, it can be concluded that these two non-synonymous SNPs decrease the susceptibility of the parasites to ART at a low level.
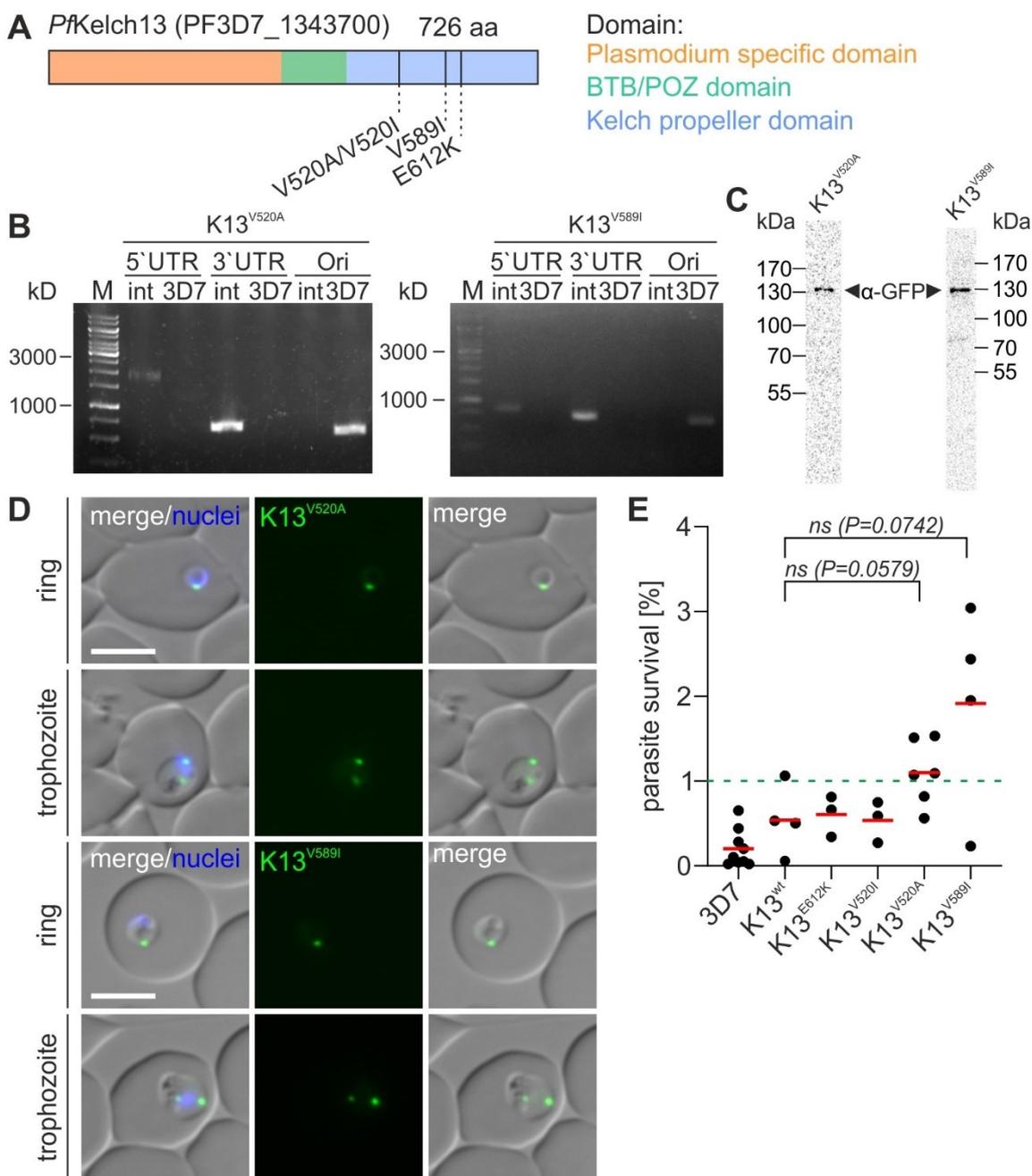
Figure 37) K13^{V520A} and K13^{V589I} render parasites resistant to ART. A) Scheme showing non-synonymous SNPs of *Pf*Kelch13 chosen for separate testing in standard RSA. **B)** Representative agarose gels showing PCR products demonstrating correct integration of the SLI plasmids into 3D7 genome to obtain K13^{V520A} and K13^{V589I} parasites. Primers (see Appendix C) were used to confirm

5`-integration (5`UTR, 1850 bp (K13^{V520A}) and 805 bp (K13^{V589I})) and 3`-integration (3`UTR, 671 bp (K13^{V520A} and K13^{V589I})) and to demonstrate absence of original locus in the K13^{V520A} and K13^{V589I} parasites (Ori, 604 bp (K13^{V520A} and K13^{V589I})). 3D7 gDNA was used as reference (ori 3D7 604 bp (K13^{V520A} and K13^{V589I})). Agarose gels of K13^{E612K} and K13^{V520I} are shown in the Appendix F.1. **C)** Western blot analysis with protein extracts of saponin-lysed parasites of K13^{V520A} and K13^{V589I} parasites. α-GFP (from mouse) was used to detect K13^{V520A} and K13^{V589I} (expected MW of 138 kDa). Molecular weight standard is indicated in kDa. Full blots are shown in Appendix F.2. **D)** Representative live cell images of K13^{V520A} and K13^{V589I} parasites. Nuclei were stained with DAPI. **E)** Graph showing parasite survival rate (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA. Each point shows an independent experiment. P value is indicated, two-tailed, unpaired Welch`s t test. *Red vertical bar* shows mean. *Green dashed line* shows 1% survival, above which parasites are considered resistant (Witkowski & Amaratunga *et al.*, 2013). *aa*, amino acid; *kD*, kilodalton; *M*, marker (GeneRuler™ 1 kb, Thermo Scientific); *int*, integrant; *ori*; original locus; *GFP*, green fluorescent protein; *wt*, wildtype; size bars, 5µm.

4.7 K13^{C580Y} parasites become more resistant to DHA after consecutively performed standard RSAs

4.7.1 ART resistant K13^{C580Y} parasites show reinvasion of fresh RBCs and less far development after DHA pulse

Different hypotheses about the behavior of ART resistant parasites after treatment with the drug exist (Kyle, 1996; Hoshen *et al.*, 2000; Teuscher *et al.*, 2010; Codd *et al.*, 2011), but so far individual surviving parasites were never visualized over time during an RSA before. To do so, a standard RSA (Witkowski & Amaratunga *et al.*, 2013) was performed with ART resistant K13^{C580Y} parasites (Birnbaum *et al.*, 2017) and after removal of DHA parasites were analyzed using long term confocal imaging (Grüring *et al.*, 2011) alongside an untreated control. Images of the same areas were taken at three different timepoints, the start after DHA removal (expected stage: rings), at 24 h (expected stage: trophozoites) and 39 hours (expected stage: schizonts or rings of next cycle) (Fig 38 A). In the untreated K13^{C580Y} control 88% of the parasites reinvaded fresh RBCs at the 39 hours` time point, whereas 12% of the parasites were late schizont stages (Fig 38 B). In contrast, the 39 hours` time point in the DHA-treated K13^{C580Y} parasites, indicated that 64% of the cells had died after the DHA pulse, whereas 33% showed further development to the late trophozoite stage and schizont stage although only one (3%) of the DHA-treated K13^{C580Y} parasites completed the cycle and produced new rings (Fig. 38 B). While it is unclear if the other parasites would have given rise to new rings at a later time point or had suffered damage from the DHA pulse or the imaging that prevented completion of the cycle, it can overall be

concluded that the DHA-treated parasites were less far developed, consistent with a growth delay of the survivors.

To better analyze this, it was assessed whether there is a developmental difference between the untreated control and the DHA-treated K13^{C580Y} parasites, by measuring the parasite size 24 hours after DHA removal (Fig 38 C). At this time point, the parasites that developed further (36%) the average size was 8µm^2, which was still significantly lower than the average size of 18 µm^2 of the untreated parasites that developed, indicating that the DHA treated parasites were delayed in growth in the first part of the cycle, possible during the DHA pulse or possibly because only more slowly developing parasites survive DHA. Further tracking of the parasites measured in Fig 38 C on their development at 39 hours showed that 3% survived DHA pulse and reinvaded new RBCs, whereas 6% arrested in ring stage phase, 8% developed into young trophozoites and 19% into late schizonts. Additionally, 25% of the quantified parasites in Fig 38 C died as ring stage and 3% as trophozoite stage parasites, whereas 36% were dead after 24 hours.

In summary, the survival of a DHA resistant K13^{C580Y} parasite was imaged for the first time and the data indicates that DHA treatment of resistant parasites delays development after the drug pulse or only the subset of more slowly growing parasites survives.
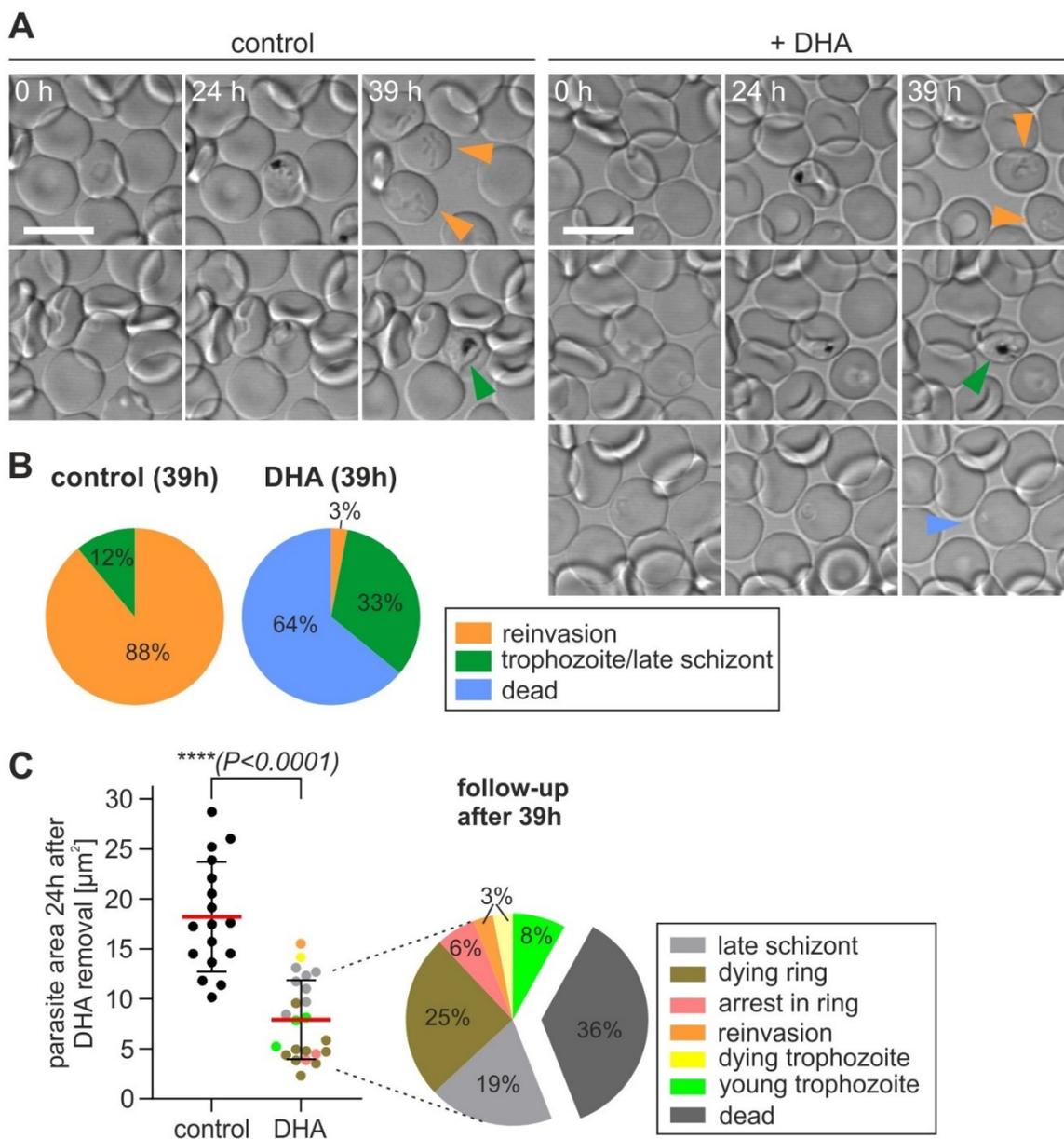
Figure 38) K13^C580Y parasites develop less far after DHA pulse. **A)** Confocal microscopy images (single z-plane of an image stack is shown) of the same K13^C580Y parasites tracked over time after removal of DHA in a standard RSA. Images of untreated control and DHA treated K13^C580Y parasites were taken at three different timepoint, directly after removal of DHA (0 h), 24 hours and 39 hours after DHA removal. **B)** Quantification of number of K13^C580Y parasites of the indicated phenotype (reinvasion: defined by disappearance of schizont and appearance of new rings in the vicinity; trophozoites/late schizont: parasites that did not complete the cycle; dead: pyknotic or dying parasites) 39 hours after DHA removal. One experiment was performed, and all parasites detected in the experiment were analyzed, for control n = 17 cells and for the DHA-treated fraction n = 36 cells were analyzed. All confocal images are shown in Appendix G.1. *Orange arrows* indicate reinvaded parasites, *green arrows* mark parasites developing into late schizonts, *blue arrows* mark dead parasites. **C)** Parasite area measurement of K13^C580Y parasites 24 hours after removal of DHA. Only the parasites that were still alive at 39 h were included in the size measurement. Parasite measured at 24 hours were followed-up at 39 hours and their fate classified into dying parasites (defined as pygnotic or vanishing parasites), arresting in the ring stage, or developing into young trophozoites / late schizonts, or reinvading parasites (indicated as percent of all parasites and depicted in a pie-

132

chart). All calculations are shown in Appendix G.2. Control n = 17 cells, DHA-treated parasites n = 36 cells. P value is indicated, two-tailed unpaired t test. Scale bar, 10µm.

4.7.2 Consecutive cycles of standard RSA render K13^{C580Y} parasites even more resistant to ART

Kelch13-based ART resistance is not a complete resistance and even in resistant cell lines only a proportion of parasites survive (Witkowski & Amaratunga *et al.*, 2013; Amaratunga *et al.*, 2014; Ariey *et al.*, 2014; Straimer *et al.*, 2015). It is unclear if the survivors harbor particular properties and if they can be selected to obtain more resistant parasites. To test whether the ART resistant K13^{C580Y} parasites (Birnbaum *et al.*, 2017) can become more resistant, a special experimental setup of consecutive cycles of standard RSAs (Witkowski & Amaratunga *et al.*, 2013) was employed to avoid the loss of resistance characteristics between RSAs, for instance if parasites with a heightened resistance display reduced growth (Fig 39 A). For this experiment, K13^{C580Y} parasites were used that derived from a cryo-stabilates of correct freshly integrated parasites, that were in culture for not longer than four to five weeks after thawing. To start the long-term-experiment, K13^{C580Y} parasites were subjected to a standard RSA. After completion of the initial RSA (Fig. 39 A, start), Giemsa smears of control (untreated K13^{C580Y} parasites) and DHA-treated K13^{C580Y} parasites were prepared at the time this is done in the RSA (72 hours post RSA initiation) to assess the proportion of surviving parasites. The DHA-treated K13^{C580Y} culture was recultivated in fresh blood and medium to continue growth of the parasites that survived the DHA pulse and when they started the next replication cycle cryo-stabilates of ring stage parasites were prepared. The parasites were grown until they reached at least 2-3 % parasitemia (typically 4 – 5 days of culture) and then tested in a subsequent RSA cycle (1st). In total, this long-term experiment was performed over 30 weeks, with one conducted RSA per week and continuous recultivation of DHA surviving K13^{C580Y} parasites after each RSA cycle.

At the beginning of the experiment, the starting K13^{C580Y} had a mean parasite survival of 12.43%. After 29 further consecutive cycles of standard RSAs each time with the DHA-survivors of the prior RSA cycle, the mean parasite survival rate had increased significantly to 39.23% (Fig 39 B). Altogether, the data indicates that ART resistant K13^{C580Y} can become even more resistant to ART after consecutive cycles of standard RSAs.
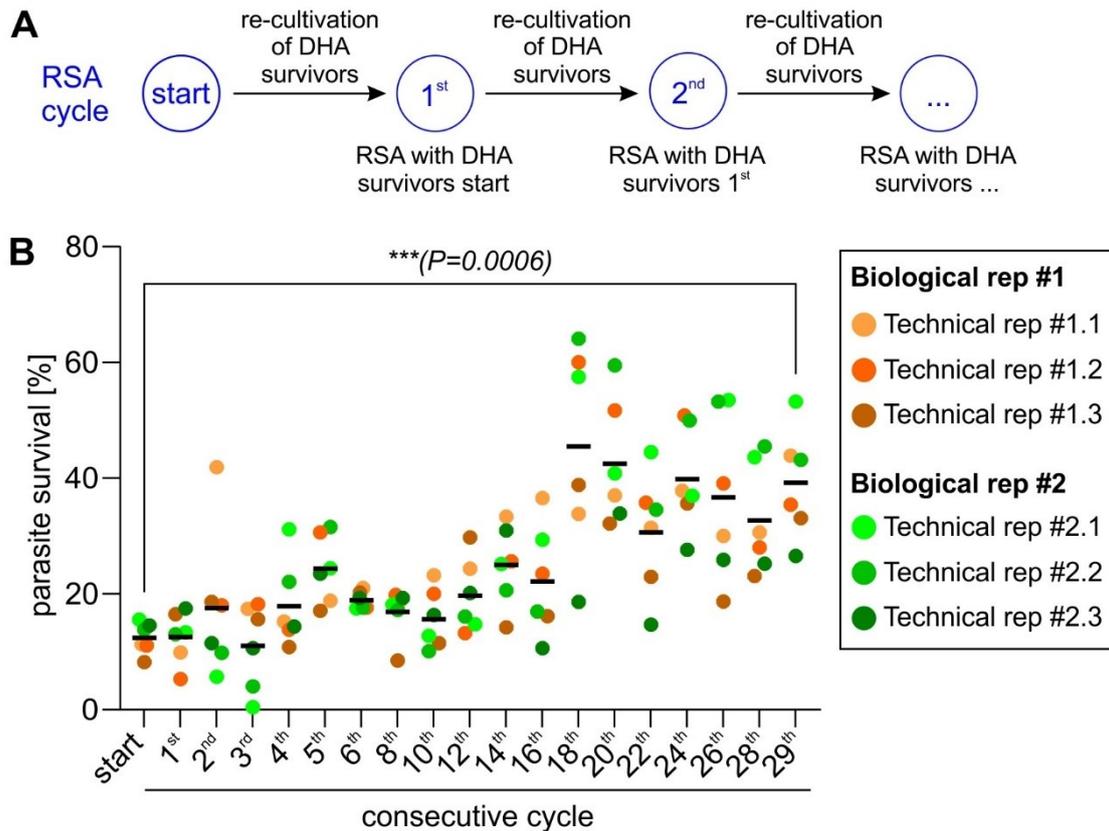
Figure 39) Consecutive cycles of standard RSA with DHA survivors render K13^{C580Y} parasites more resistant over time. A) Scheme of experimental procedure of consecutive standard RSA cycles performed with DHA survivors of the respective prior cycle. **B)** Graph showing parasite survival rate of K13^{C580Y} (% survival compared to control without DHA) 72 hours after 6 h DHA treatment in standard RSA (Witkowski & Amaratunga *et al.*, 2013). Six experiments per cycle were performed consisting of two biological and three technical replicates (see color code). P value is indicated, two-tailed, Welch´s t test. *Red bars* show mean.

4.8 Non-synonymous SNPs of *pfkelch13* influence protein amount and parasite fitness

4.8.1 Non-synonymous SNPs causing ART resistance influence *Pf*Kelch13 protein amount

Previous studies showed, that the C580Y mutation influences the abundance of the *Pf*Kelch13 protein present in the cell, thus determining the endocytosis rate and consequently regulating ART resistance (Birnbaum *et al.*, 2020). This data raised the question whether, and to which extent, other non-synonymous SNPs modulate the

*Pf*Kelch13 protein amount. To test this, the *Pf*Kelch13 amount in the cell lines with mutations here found to confer moderate resistance, K13^{V520A}, K13^{C580Y} (section 4.6.5) and the highly resistant K13$^{C580Y\_29th}$ of the consecutive RSA experiment (section 4.7.2) was measured and compared to K13^{wt} and non RSA-selected K13^{C580Y} parasites. Here again, K13^{C580Y} as well as K13^{V520A} parasites were used that derived from cryo-stabilates of correct freshly integrated parasites that were in culture for not longer than four to five weeks after thawing (see section 3.3.4). To start the experiment, all parasite lines were synchronized twice, using sorbitol, and *Pf*Kelch13 protein amount was assessed by recording the GFP fluorescence intensity of *Pf*Kelch13 foci in ring stage parasites using ImageJ with microcopy images (acquired using identical settings) of live cells (Fig 40 A).

In comparison to *Pf*Kelch13 foci in the K13^{wt} parasites (mean fluorescence intensity 0.038, calculated average from three independent experiments where total fluorescence of GFP signal was measured), the total fluorescence of the foci in K13^{V520A} parasites was reduced by 16% (mean fluorescence intensity 0.032). The *Pf*Kelch13 foci in K13^{C580Y} parasites showed 56% less total fluorescence (mean fluorescence intensity 0.017) compared to K13^{wt} control, whereas the highly resistant K13$^{C580Y\_29th}$ cell line showed the highest reduction of 70% (mean fluorescence intensity 0.011). When the highly resistant K13$^{C580Y\_29th}$ cell line was compared to its original K13^{C580Y} parasite line, the *Pf*Kelch13 protein amount was reduced by 31%.

The resistance level of the different *Pf*Kelch13 cell lines negatively correlated with the *Pf*Kelch13 protein amount present (Fig 40 B). The higher the parasite survival of the respective parasites in standard RSA (Witkowski & Amaratunga *et al.*, 2013) was, the lower the amount of *Pf*Kelch13 protein in the cell (Fig 40 B). The K13$^{C580Y\_29th}$ cell lines which had a survival rate of 39.23% showed the lowest protein amount, while the sensitive K13^{wt} parasites (mean survival rate of 0.54% in RSA) showed the highest *Pf*Kelch13 levels. The K13^{V520A} parasite line which had a mean survival rate of 1.10% in standard RSA, showed protein levels between K13^{wt} and K13^{C580Y}.

In summary, these data show that different non-synonymous SNPs influence the *Pf*Kelch13 protein amount per focus in rings (and hence per cell) and that parasite susceptibility to ART inversely correlates with protein amount present in the cell.
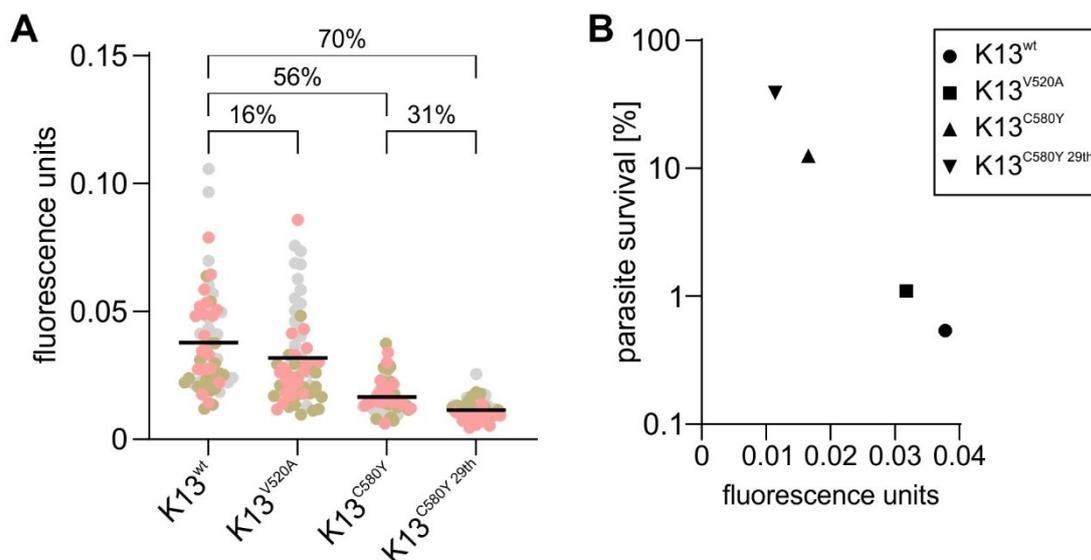
Figure 40) Resistance-causing non-synonymous SNPs reduce *Pf*Kelch13 protein amount. A) Quantification of *Pf*Kelch13 protein amount expressed by the different mutant measured by GFP-fluorescence intensity. Total fluorescence intensity of individual *Pf*Kelch13 foci of young ring stage parasites was measured by subtracting background signal (cytosolic fluorescence) from detected fluorescence signal, multiplied with the focus area. Three independent experiments were performed represented by the colors, and per experiment n = 20 cells were recorded. Numbers indicate the *Pf*Kelch13 protein reduction between K13[wt] control and the different mutants. *Black bars* indicate mean. **B)** Mean parasite survival rate in standard RSAs (Witkowski & Amaratunga *et al.*, 2013) plotted against *Pf*Kelch13 protein amount measured by GFP-fluorescence intensity (log scale). For this comparison, the RSA data from section 4.6.5 and 4.7.2 were used where the mean parasite survival rate was calculated from at least three independent experiments per cell line. *wt*, wild type.

4.8.2 Parasite fitness is influenced by different non-synonymous SNPs and negatively correlates with ART resistance

Previously, it was shown that ART resistant K13[C580Y] parasites have a reduced fitness (Rosenthal, 2013; Hott *et al.*, 2015; Nair *et al.*, 2018; Stokes *et al.*, 2021). In order to test if the parasites carrying with the non-synonymous SNP that results in only moderate ART resistance have a higher fitness than the more resistant K13[C580Y] parasites. It was therefore here tested how the fitness level of the K13[V520A] cell line compares to that of the K13[C580Y] and the K13[wt] parasites.

To assess the fitness cost of K13[wt], K13[V520A] and K13[C580Y] parasites, a GFP fluorescence-based fitness assay was performed where the different cell lines were co-cultivated in a 1:1 ration with 3D7 parasites. This took advantage of the fact that 3D7 parasites do not contain GFP and are therefore easily discerned from the transgenic parasites. The co-cultures were

grown until one of the different cultures overgrew the other co-culture (cut-off of less than 5 % of all parasites). In regular intervals, the proportions of 3D7 to K13^{wt}, K13^{V520A} and K13^{C580Y} parasites was measured by live cell imaging and the percentage of GFP positive cells in the respective culture was recorded (Raw curves of individual experiments shown in Appendix H.1 and H.3). To estimate the relative growth based on the individual fitness, a non-linear regression was performed, resulting in a formula that was used to estimate the fitness cost per generation (Fig 41 A). Additionally, the fitness assay was conducted in a growth medium containing a limiting amount of amino acids (Low AA medium) to test for the additional influence of low nutrients.

In comparison to K13^{wt}, which had a mean fitness cost per generation of 0.015 in RPMI complete medium and 0.017 in low AA medium, the K13^{V520A} parasites had an increased fitness cost of 0.21 in RPMI complete medium and 0.14 in a medium with a limiting amount of amino acids. The K13^{C580Y} parasites had the highest fitness cost per generation compared to the K13^{wt} control with 0.30 in RPMI complete and 0.39 in low AA medium (Fig 41 B). It should be noted that there was some variation in the individual experiments for the K13^{V520A} parasites that either leaned more closely to the fitness of the K13^{wt} control or that of the K13^{C580Y} parasites (Appendix H.1 and H.3). Nevertheless, its placement between the two parasite lines in terms of fitness was consistent.

When the fitness cost of the different *Pf*Kelch13 cell lines were compared to the respective parasite survival rates of the standard RSA (Witkowski & Amaratunga *et al.*, 2013), a positive correlation was observed (Fig 41 C), and hence also inversely correlating with the *Pf*Kelch13 protein amount per cell. K13^{C580Y}, which had the highest RSA survival rate of the tested cell lines had the highest fitness costs, while the K13^{V520A} cell line displayed a moderate ART resistance level and a lower fitness cost.

Altogether, the fitness experiments showed that the non-synonymous SNPs C580Y and V520A of *Pf*Kelch13 reduce the fitness of the parasite. It was also shown that the fitness deficiency is higher in the highly resistant K13^{C580Y} than in the moderately resistant K13^{V520A} parasites. These data suggest that non-synonymous SNPs present in Africa cause lower levels of ART resistance but may be advantageous for the parasite since their fitness is less impaired which might be a benefit in endemicity settings with frequent multiple infections and lower rate of ART treatment due to semi-immunity.
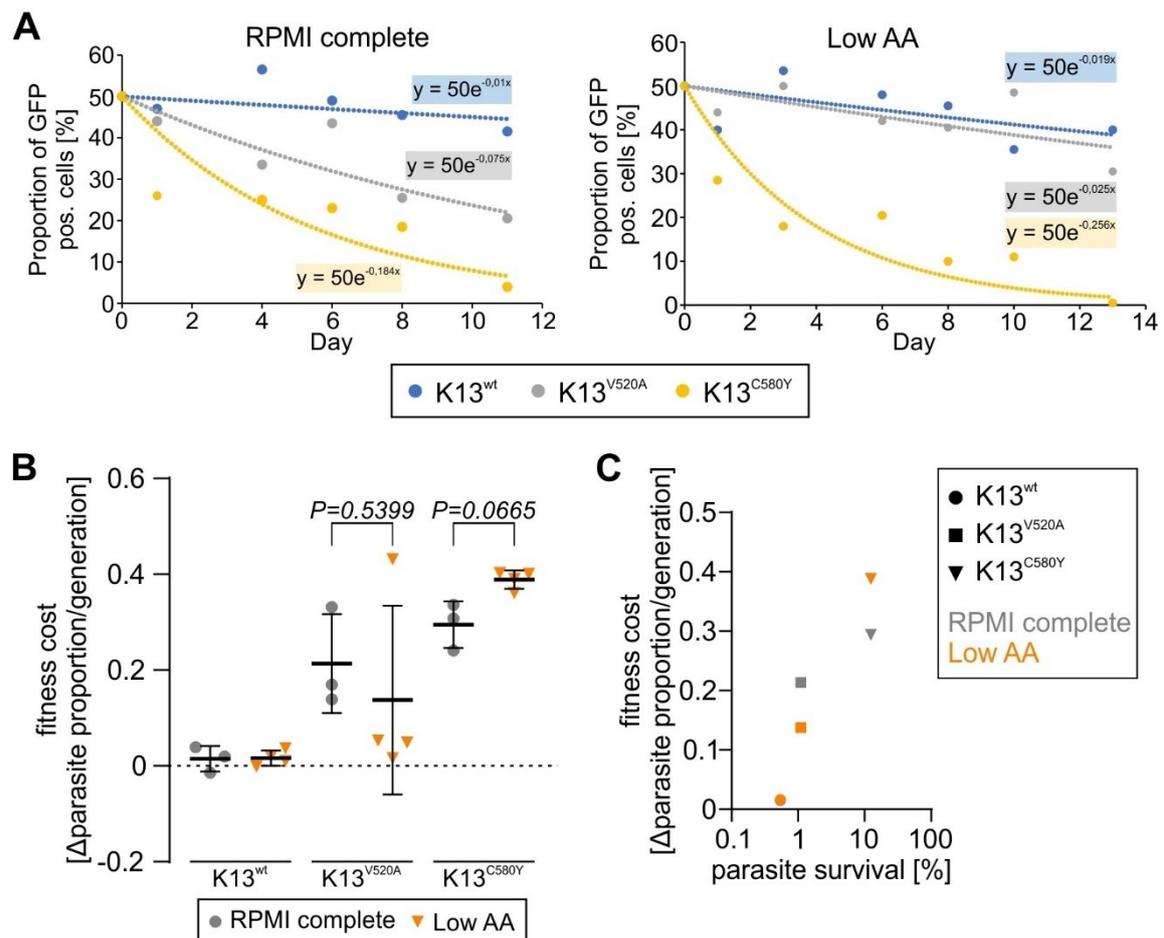
Figure 41) Higher ART resistance level results in higher fitness cost. A) Competitive GFP-based fitness assay performed in RPMI complete medium and a medium with limited amount of amino acids (low AA). Proportional growth of K13^{wt}, K13^{V520A} and K13^{C580Y} parasites compared to 3D7 wild type parasites grown in the same culture. One representative non-linear regression curve per condition (RPMI complete or Low AA) is depicted deriving from respective raw curves expressing the proportional amount of GFP positive parasites per total parasitemia. Fitness cost per generation of the different strains were calculated based on the indicated functions (raw curves in Appendix H.1 and H.3). **B)** Fitness cost per generation of the different *Pf*Kelch13 strains in RPMI complete and low AA medium (calculations see Appendix H.2 and H.4). For this, in a first step the trendline factor from the functions indicated in (A) was duplicated (assumption that one generation corresponds to two days), and growth factor per generation was differentiated. Fitness cost per generation were calculated by subtracting the differentiated value from 1 (1 representing 100% fitness). Black bars indicate mean fitness cost per generation. Three independent experiments with two technical replicates were performed in RPMI complete medium and 4 independent experiments with two technical replicates in low AA medium. P values are indicated, two-tailed Welch´s t test. **C)** Fitness cost per generation of K13^{wt}, K13^{V520A} and K13^{C580Y} parasites in RPMI complete and low aa medium as assessed in (A) and plotted against mean parasite survival (log scale) in RSA (Witkowski & Amaratunga *et al.*, 2013). For this comparison, the RSA data from section 4.6.5 and 4.7.2 were used where the mean parasite survival rate was calculated from at least three independent experiments per cell line. *wt*, wild type.

5 Discussion

5.1 *Pf*Kelch13-independent ART resistance

Previous work demonstrated that ART resistance is mediated either in a *Pf*Kelch13-dependent manner or can also be caused by a *Pf*Kelch13-independent mechanism (Mukherjee *et al.*, 2017). To identify interacting partners of *Pf*Kelch13, a modulated version of the proximity dependent biotin identification (BioID) was used, called dimerization-induced quantitative BioID (DiQ-BioID) (Birnbaum *et al.*, 2020). This newly developed BioID technique, in combination with quantitative mass spectrometry, helped define a novel Kelch13 compartment in the parasite. This analysis resulted in a list of high confident hits that were mostly of unknown function and were named KICs. Interestingly, the list also included proteins which had been suspected in ART resistance before or were detected by genome-wide association studies as potential resistance markers, such as *Pf*UBP1, *Pf*Eps15, *Pf*KIC6 and *Pf*MyosinF (Hunt *et al.*, 2007; Hunt *et al.*, 2010a; Borrmann *et al.*, 2013; Henriques *et al.*, 2014; Cerqueira *et al.*, 2017). These findings indicated that the identified hits define a pathway together with *Pf*Kelch13 that is relevant for ART resistance. More than 10 members of the Kelch13 compartment were validated as true interactors of *Pf*Kelch13 whereas two candidates, *Pf*MCA2 (PF3D7_1438400) and *Pf*MyosinF (PF3D7_1329100; previously annotated as *Pf*MyosinC), had not previously been validated to co-localize with *Pf*Kelch13. One aim of the thesis was to functionally characterize these two candidates as well as confirm their co-localization with *Pf*Kelch13 to validate them as true members of the Kelch13 compartment.

5.1.1 *Pf*MCA2 belongs to the Kelch13 compartment and truncated versions of the protein mediate ART resistance *in vitro*

Only little information exists about the functional role of *Pf*MCA2 in *Plasmodium* parasites despite the fact that metacaspases represent an interesting antimalarial target since metacaspases are not present in humans. Metacaspases are cysteine-dependent proteases that are distantly related to metazoan caspases, having a Cys-His catalytic dyad but different substrate specificity (Uren, 2000; Tsiatsiani *et al.*, 2011). They are classified into three different types based on structural features (Tsiatsiani *et al.*, 2011). Type I metacaspases are found in protozoans and fungi, while plants express both, type I and II

metacaspases. In plants, type I metacaspases represent the dominating class. Type I metacaspases contain an N-terminal pro-domain comprising a proline-rich repeat motif and a zinc finger motif (Vercammen *et al.*, 2004; Tsiatsiani *et al.*, 2011). The currently identified type III metacaspases are exclusively found in algae which have undergone secondary endosymbiosis (Klemenčič & Funk, 2018b). Irregular distribution of homologues across different phyla hinders the functional specification of metacaspases. In the plant *Arabidopsis thaliana,* a total of nine different metacaspases were identified (Cox, 2011). Looking at the protozoan *Leishmania*, a single metacaspase is encoded in all species, except for *L. donovani* and *L. infantum* subtypes which express two different metacaspases (Meslin & Zalila *et al.*, 2011). In contrast, *Trypanosoma brucei* encodes five different redundant metacaspases (*Tb*MCA1 – 5). The *Trypanosoma* metacaspases were shown to likely be involved in the regulation of the programmed cell death (Szallies *et al.*, 2002) which is similar to *Leishmania* metacaspases (Casanova *et al.*, 2015) and is a central role of metazoan caspases. Additionally, *Trypanosoma* metacaspases are enrolled in cytokinesis (Helms *et al.*, 2006), and cell proliferation (Meslin & Zalila *et al.*, 2011). For *Plasmodium* parasites, three different metacaspases were reported in *P. falciparum* (*Pf*MCA1 (PF3D7_1354800); *Pf*MCA2 (PF3D7_1438400) and *Pf*MCA3 (PF3D7_1416200)), *P. vivax* (*Pv*MCA1-3) and the murine species *P. berghei* (*Pb*MCA1-3). Earlier studies suggested a role for *Plasmodium* metacaspases in stress response-related programmed cell death, and in the regulation of growth and development (Picot *et al.*, 1997; Meslin *et al.*, 2007; Ch'ng *et al.*, 2010; Rathore *et al.*, 2015). Nevertheless, the specific roles of these metacaspases are unknown and it is difficult to make predictions about function based on the similarity to metacaspases in other organisms (Shrestha & Megeney, 2012; Vandana *et al.*, 2019). Hence, there is little that can be drawn from metacaspases in other organisms to define the function of *Pf*MCA2 and its potential role in the Kelch13 compartment in more detail. *Pf*MCA2 also possesses a Cys-His catalytic dyad which shows its structural similarity to metazoan caspases (Tsiatsiani *et al.*, 2011). This metacaspase belongs to the C14 family of clan CD proteases, comprising two catalytic domains, a large p20 subunit and a small p10 subunit (Klemenčič & Funk, 2018a). In a recent publication, *Vandana et al.* performed experiments to elucidate the enzymatic activity and substrate specificity of *Pf*MCA2 (Vandana *et al.*, 2018). This work showed that *Pf*MCA2 has an arginine/lysine substrate specificity and cleaves *Pf*TSN, which is an essential functional component of the spliceosome Sm core complex (Rathore *et al.*, 2015). Additionally, it was suggested that *Pf*MCA2 plays a potential role in stress response related cell death (Vandana *et al.*, 2018; Vandana *et al.*, 2020).

Prior attempts to tag the full-length *Pf*MCA2 C-terminally with a sequence coding for GFP and 2xFKBP-GFP failed (personal communication, Jakob Birnbaum). In this work, using the

SLI system (Birnbaum *et al.*, 2017), a sequence encoding a 3xHA was fused successfully to the C-terminus of the full-length *pfmca*2 endogenous locus for the first time (section 4.1.1). This allowed the localization of full length *Pf*MCA2 in blood stage parasites, demonstrating that it is expressed throughout the entire asexual cycle. In ring stage parasites, *Pf*MCA2 was found in one focus, while in trophozoite stage parasites one or more foci were detected. These foci were either located close to the parasite food vacuole or to vesicles in the cell periphery. In schizont stages, *Pf*MCA2 was more difficult to detect as the signal was more diffuse but nevertheless these parasites usually had one focus close to the food vacuole (section 4.1.1). Besides, the MCA2^wt^-3xHA cell line, a GFP-tagged MCA2 cell line was generated, were *pfmca2* was disrupted at amino acid position 1344 (MCA2^Y1344Stop^). The underlying nucleotide change represents a SNP that is listed in MalariaGEN to be present in Africa and SEA. Live cell imaging revealed a similar localization of MCA2^Y1344Stop^ as wild type *Pf*MCA2 in the 3xHA tagged cell line (section 4.1.3). A localization of *Pf*MCA2 in the parasite cytoplasm is supported by the findings of *Vandana et al.* who showed that *Pf*MCA2 requires a neutral pH, indicating that it is likely functional in the cytoplasm or in organelles with neutral pH (Vandana *et al.*, 2018). Analysis of mRNA transcript level in *P. falciparum* revealed that *Pf*MCA2 is mainly expressed in schizonts and gametocytes, while ring and trophozoite stage parasites show a lower abundance of *Pf*MCA2 mRNA transcripts (Young *et al.*, 2005; Bártfai *et al.*, 2010; Toenhake *et al.*, 2018; Vandana *et al.*, 2018). However, the work in this thesis clearly shows that *Pf*MCA2 is expressed in rings and trophozoites. The expression and localization of *Pf*MCA2-3xHA in schizont and gametocyte stages needs to be analyzed in more detail, and in a next step the signal intensity could be compared between ring, trophozoite, schizont and gametocyte stage parasites via fluorescence microscopy or western blot analysis to determine the stage-specific protein levels which often deviate from that of the transcripts (Foth *et al.*, 2011).

DiQ-BioID data indicated that *Pf*MCA2 is present at the Kelch13 compartment. To clarify this finding, co-localization experiments with the MCA2^wt^-3xHA cell line transfected with an episomal Kelch13 construct were performed and demonstrated that both *Pf*MCA2 and *Pf*Kelch13 co-localize (section 4.1.2). The data was additionally confirmed by live cell imaging of the MCA2^Y1344Stop^ cell line transfected with an episomal Kelch13 construct (section 4.1.4). These experiments also showed that the truncation did not alter the localization of *Pf*MCA2 or interfered with the relative positioning of *Pf*MCA2 to *Pf*Kelch13. To verify that the co-localization between both proteins also means that they interact would require co-immunoprecipitation experiments.

It was previously shown that it is possible to disrupt the original genomic locus of the protein at amino acid position 57 via SLI-TGD and express a truncated version of the *Pf*MCA2

(Birnbaum *et al.*, 2020). Determination of essentiality of *Pf*MCA2 for parasite growth via SLI-TGD and flow cytometry-based growth rate analysis demonstrated, that *Pf*MCA2 is dispensable for parasite development during the asexual replication, although some reduction in growth was apparent when compared to wild type 3D7 (Birnbaum *et al.*, 2020). However, this truncation rendered parasites less susceptible to ART, indicating that *Pf*MCA2 has a role in the pathway involved in ART resistance and that other changes in this gene could be important for Kelch13-independent resistance in the field. In correlation to these results, *in vitro* deletion of *Pf*MCA2 in 3D7 parasites by a non-synonymous mutation, listed in MalariaGEN at position 1344, leading to the amino acid change Y1344Stop compared to 3D7 sequence, was successful (section 4.1.3). The corresponding SNP, T to A at position 4032 of the coding DNA, is found in African field isolates with a mean prevalence of 52%, and in SEA with 5% mean prevalence. *In vitro* deletion of *Pf*MCA2 in 3D7 parasites at position 1344 was successful and when tested in RSA, the MCA2[Y1344Stop] displayed a reduced susceptibility to ART, indicating that although this protein was still correctly localized, it had been inactivated sufficiently to mimic the result with the truncation at amino acid position 57 (see (Birnbaum *et al.*, 2020); section 4.1.3). Performing a motif scan, using the Expasy Swiss Bioinformatics Resource Portal, to predict the different domains of *Pf*MCA2 revealed that the putative catalytic caspase domains are located at amino acid positions 1546 – 1626 (E-value $6.1e^{-05}$) and 1546 – 1777 (E-value 0.0012) (Sigrist *et al.*, 2010). This means that by both truncations, either by targeted gene disruption using SLI or deleting the original *pfmca2* locus through the Y1344Stop mutation, resulted in the loss of the catalytical caspase domain. Interestingly, the truncation at amino acid position 57 achieved a higher parasite survival rate (mean survival rate ~5%), while the truncation at amino acid position 1344 resulted in a moderate survival rate (mean survival rate 1.64%) (section 4.1.3). This suggests that the truncation at amino acid position 1344 does potentially less inactivate *Pf*MCA2, resulting in a lower ART resistance level.

To test whether the non-synonymous SNP is relevant in the field, RNA sequence data from African field isolates were analyzed. In 13 analyzed samples, the mutation was found (position 4032 of coding DNA sequence; data kindly provided by Anna Bachmann). Interestingly, by analyzing the RNA seq data a second mutation was found two base pairs upstream, in the same codon, at position 4030 of the coding DNA sequence, resulting in the codon change Y1344K compared to 3D7 reference sequence. This second detected mutation (position 4030) was simultaneously present together with the SNP in position 4032 in all 13 samples, resulting in an amino acid change and maintenance of the reading frame, not a truncation, indicating that the Y1344Stop change likely does not occur in the field and likely is not relevant for resistance *in vivo*.

Functional data about the specific role of *Pf*MCA2 in the Kelch13 compartment and its involvement in ART resistance are still missing. Experiments conducted in yeast models showed that the metacaspase YCA1 is responsible for the regulation of the cellular stress response and maintenance of protein homeostasis in *S. cerevisiae* (Lee & Brunette *et al.*, 2010; Meslin & Zalila *et al.*, 2011). In *P. falciparum* it was demonstrated that the redundant metacaspase *Pf*MCA1 is a main regulator of oxidative stress response, causing apoptosis-like cell death, organelle dysfunction and disruption of cellular homeostasis (Picot *et al.*, 1997; Meslin & Beavogui *et al.*, 2011; Rathore *et al.*, 2015). In 2018, *Vandana et al.* showed that *Pf*MCA2 is potentially involved in programmed cell death and functional studies indicated an increased oxidative stress after treatment with an effector caspase inhibitor in *P. falciparum* due to a downregulation of *Pf*MCA2 (Vandana *et al.*, 2018). This work further tested whether *Pf*MCA2 is involved in the cleavage of hemoglobin, similar to other cysteine proteases such as falcipains or plasmepsins, but found no interaction between hemoglobin and the metacaspase, suggesting a lack of suitable binding sites (Vandana *et al.*, 2018). Besides this data, a study performed in *Aspergillus fumigatus* showed that fungal metacaspases are involved in ER-related stress response and regulate the UPR pathways in fungi (Richie *et al.*, 2007). Altogether the findings suggest that *Pf*MCA2 is potentially involved in the regulation of oxidative stress response and the UPR pathway. Since an increased cellular stress response as well as an upregulated UPR pathway was detected in in ART resistant parasites, this would provide a link between *Pf*MCA2, *Pf*Kelch13, and ART resistance (Dogovski *et al.*, 2015; Mok *et al.*, 2015). To further investigate this in *P. falciparum*, the level of reactive oxygen species in MCA2^{wt}-3xHA and MCA2^{Y1344Stop} parasites could be analyzed and compared. This could be achieved by analyzing the genomic expression pattern of wildtype *Pf*MCA2 and comparing it to the *Pf*MCA2 transcription level of the different truncated versions in the presence or absence of oxidative stress. This could provide insight into stress response mechanisms, as analysis of altered genetic expression in yeast, mammalians, and Drosophila helped to define such processes in more detail in these organisms (Nadal *et al.*, 2011). Additionally, N-terminal tagging of *Pf*MCA2 might permit to obtain a full length GFP and FKBP tagged version which could be used to conditionally inactivate it. This could be used to obtain direct functional insights into the role of *Pf*MCA2 in *P. falciparum*. Similar to *Pf*Kelch13, DiQ-BioID experiments combined with quantitative mass spectrometry would help to unravel the interactome of *Pf*MCA2 and further confirm the co-localization with *Pf*Kelch13 and the Kelch13 compartment. Such experiments could provide a link of the stress response pathways metacaspases are suspected to be involved with endocytosis and resistance to ART.

5.1.2 *Pf*MyosinF is an actin-associated protein that is part of the Kelch13 compartment and contributes to hemoglobin uptake in *P. falciparum* parasites

*Pf*MyosinF was indicated by the DiQ-BioID data as potential interaction partner of *Pf*Kelch13 (Birnbaum *et al.*, 2020). As neither the presence of *Pf*MyosinF in the Kelch13 compartment nor a true interaction to *Pf*Kelch13 was so far verified and only little information exists about its functional role in *P. falciparum*, *Pf*MyosinF was investigated in this work.

To assess the co-localization of *Pf*MyosinF with *Pf*Kelch13, a cell line where the native *Pf*MyosinF locus was modified to tag this protein with 2xFKBP-GFP-2xFKBP (MyoF^{wt}-2x2 cell line, kindly provided by Ernst Jonscher), was transfected with an episomally expressed mCherry-*Pf*Kelch13 construct (section 4.2.1). Live cell images of the original MyoF^{wt}-2x2 cell line showed that these parasites spontaneously developed vesicles in trophozoite and schizont stages that were not (or less frequently) present in the 3D7 parent. Thus, a second integrant cell line was newly established as control where the full-length *Pf*MyosinF protein was tagged with a sequence coding for 3xHA (MyoF^{wt}-3xHA parasites). Imaging revealed that this cell line did not develop spontaneous vesicles in trophozoite and schizont stages. These parasites were transfected with an episomally expressed mCherry-*Pf*Kelch13 construct. Immunofluorescence assays using the MyoF^{wt}-3xHA+K13 cell line and live cell imaging of the MyoF^{wt}-2x2+K13 parasites showed only limited co-localization of *Pf*MyosinF with *Pf*Kelch13 but revealed that *Pf*MyosinF was mostly found in close proximity to the Kelch13 compartment (section 4.2.1). This was observed in both, Formaldehyde/glutaraldehyde-fixed MyoF^{wt}-3xHA+K13 parasites and MyoF^{wt}-2x2+K13 parasites, indicating that the vesicular phenotype in trophozoite and schizont stages did not influence the relative localization of both proteins. Performing an IFA with acetone-fixed MyoF^{wt}-3xHA+K13 parasites resulted in the detection of more partially co-localizing and closely localizing *Pf*MyosinF and *Pf*Kelch13 foci (see section 4.2.1). This indicated that either the fixation method influenced the accessibility of antigen epitopes by the detecting antibodies, resulting in the loss of some closely localizing foci in formaldehyde/glutaraldehyde-fixed parasites. Alternatively, the fixing method may have affected the spatial arrangement of closely apposed compartments or may have led to a partial loss of integrity of the compartments which may have resulted in the observed differences. Nevertheless, the co-localization of *Pf*MyosinF and *Pf*Kelch13, or rather the close association, were clearly apparent with both methods. Overall, the failure of the two proteins to fully overlap in most instances were unexpected since *Pf*MyosinF was found in the DiQ-BioID hit list, although with lower enrichment and probability than the hits that were so far validated to belong to the Kelch13 compartment.

To functionally characterize the *Pf*MyosinF protein, knock sideways experiments using the MyoF^wt-2x2 cell line transfected with an episomal nuclear mislocalizer (MyoF^wt-2x2+1xNLS, kindly provided by Ernst Jonscher) were performed to assess the essentiality of the protein for asexual replication. Flow cytometry-based growth assays, as wells as live cell imaging revealed that inactivation via mislocalization of *Pf*MyosinF into another cell compartment was only partially achieved but nevertheless significantly reduced parasite growth (section 4.3.1). Fluorescence microscopy showed that *Pf*MyosinF is only weakly expressed in ring stages, while in later developmental stages one *Pf*MyosinF foci was always localized close to the food vacuole and two, or sometimes more foci, were detected close to vesicles (based on appearance in DIC) in the cell periphery (section 4.3.1). These findings suggest that *Pf*MyosinF may be involved in endocytic processes and potentially supports the *Pf*Kelch13-mediated hemoglobin uptake (Birnbaum *et al.*, 2020).

In general, myosins represent molecular motor proteins which are dependent on actin. Most eukaryotic organisms are reliant on this actomyosin motor for various cellular functions such as organellar or vesicle transport, cell movement, and mitotic processes (Raposo *et al.*, 1999; Hasson, 2003; Salas-Cortes *et al.*, 2005; Arden *et al.*, 2007; Woolner *et al.*, 2008; Rump *et al.*, 2011; Hartman & Spudich, 2012; Carmeille *et al.*, 2021). To assess the relative position of *Pf*MyosinF to actin filaments, the MyoF^wt-2x2 cell line was transfected with an episomal construct expressing an actin-chromobody (a kind gift of Markus Meissner; (Periz *et al.*, 2017)). Live cell imaging revealed that filamentous actin accumulated close to the *Pf*MyosinF foci which were present at the FV or the vesicular structures at the cellular periphery (section 4.3.4). This finding indicates that *Pf*MyosinF is part of the actomyosin motor in *P. falciparum* and that *Pf*MyosinF together with actin contribute to endocytic vesicular trafficking. Further experiments need to be performed to clarify the association of *Pf*MyosinF and actin. A next step would be to observe the relative position of *Pf*MyosinF and actin upon the inactivation of *Pf*MyosinF via the knock sideways system.

Myosins consist of three main domains. The N-terminal head domain, which is conserved across different phyla, is responsible for ATPase activity and for the generation of moving forces mediated via the binding of the myosin heavy chain to actin filaments. The head domain is used to classify myosins into different classes. The second domain is the short neck which interacts with the myosin light chain. The third domain, the C-terminal tail domain, regulates the binding to different cargoes or other proteins defining the specificity of the myosin protein (Foth *et al.*, 2006). *Pf*MyosinF belongs to the class XXII myosins. In the C-terminal tail region this protein contains five WD40 repeats that are known to mediate the binding to other proteins, resulting in the formation of multiprotein complexes (Stirnimann *et al.*, 2010). Since the data presented here in this work may indicate an

interaction of *Pf*MyosinF and *Pf*Kelch13 (section 4.2.1), this characteristic domain feature would suggest the hypothesis that *Pf*MyosinF is interacting with *Pf*Kelch13 and its compartment members via the WD40 repeats-containing tail region, thereby regulating the Kelch13 compartment controlled endocytic processes. In this work, it was seen that C-terminally tagging of *Pf*MyosinF resulted in a partial phenotype, leading to the accumulation of vesicles in later developmental stages (section 4.3.4). This suggests that the C-terminus, might be important for vesicular trafficking to the food vacuole, which is impaired when the C-terminus is altered by tagging. Additionally, it was seen in live cell images of the MyoF^wt-2x2+K13 cell line that ~44% of the *Pf*MyosinF foci did not co-localize to *Pf*Kelch13, suggesting that C-terminal tagging here potentially affected the interaction between both proteins (section 4.2.1). To further investigate this theory, binding assays would need to be performed to define the potential binding of *Pf*Kelch13 and its compartment members to the different *Pf*MyosinF domains. Additionally, the interaction with other proteins needs to be clarified, e.g. using the DiQ-BioID approach to see whether *Pf*MyosinF interacts with further members of the Kelch13 compartment. Alternatively, it is also possible that the influence of *Pf*MyosinF on the endocytosis process is less direct and involves transport of Kelch13 compartment-derived endocytic structures to the FV rather than a direct interaction to *Pf*Kelch13. In that case the contact to *Pf*Kelch13 and its compartment proteins may be more spurious or short-lived.

In a further step it was analyzed whether the spatial arrangement of *Pf*MyosinF and *Pf*Kelch13 foci depends on actin. To test this, an inhibitor of actin polymerization, Cytochalasin D, was used to see whether the inhibition influences the relative position of *Pf*MyosinF and *Pf*Kelch13 foci (section 4.2.2). Treatment of the MyoF^wt-2x2+K13 parasites for one hour with Cytochalasin D resulted in the dissociation of *Pf*MyosinF and *Pf*Kelch13 foci in trophozoite stages and the dispersion of the *Pf*MyosinF signal in late trophozoites. This data indicates that upon Cytochalasin D treatment the actomyosin motor is impaired, leading to a disruption of the spatial arrangement of the Kelch13 compartment and *Pf*MyosinF. To further verify these findings, the experiment needs to be repeated with more cells. Furthermore, a synchronous culture could be used to better assess the effect since this preliminary experiment was performed with an asynchronous culture.

Recent data demonstrated that inactivation of *Pf*Kelch13 and its compartment members results in ART resistance due to reduced hemoglobin uptake and thus reduced ART activation (Birnbaum *et al.*, 2020). The data obtained here indicates that *Pf*MyosinF is found close to *Pf*Kelch13 in the parasite cell and that its function as motor protein may be involvement in endocytosis. To test whether *Pf*MyosinF is involved in hemoglobin uptake, a bloated food vacuole assay was performed (section 4.3.3). In this assay, a bloated food

vacuole phenotype is detectable when hemoglobin uptake is operational. The experiment revealed that upon inactivation of *Pf*MyosinF by knock sideways, endocytic uptake of hemoglobin was reduced (section 4.3.3). This finding suggests that *Pf*MyosinF is important for the transport of hemoglobin containing vesicles to the food vacuole, which is impaired when *Pf*MyosinF is partially inactivated. Comparison of the vesicle number between control parasites and rapalog-cultured parasites showed, that the control of the MyoF^wt-2x2+1xNLS cell line already had ~ 5 vesicles per cell (average number of three independent experiment), whereas the parasites where rapalog was added had ~ 8 vesicles per cell (average number of three independent experiments). Prior experiments in a parasite line where *Pf*VPS45 (a protein known to be involved in host cell cytosol uptake) can be conditionally inactivated the control parasites had ~ 2 vesicles per cell, while the parasites with rapalog (*Pf*VPS45 inactivation) displayed ~ 10 vesicles per cell (Jonscher *et al.*, 2019). This indicated that already the tagging of *Pf*MyosinF resulted in a partial phenotype as these parasites had an increased number of vesicles compared to the baseline of the VPS45 cell line. To further clarify whether this is an artificial effect caused by the C-terminal tagging of the full-length *Pf*MyosinF with 2xFKBP-GFP-2xFKBP, the experiments need to be repeated using 3D7 parasites as a control in parallel. Additionally, the MyoF^wt-3xHA cell line could be used as another control since this cell line did not appear to display an increased number of vesicles during normal culturing.

Another hint to the involvement of *Pf*MyosinF in endocytosis or vesicular trafficking is based on its C-terminal WD40 repeat domains. Prior data obtained in mammalian cells indicated that WD40 domains can bind to phosphoinositides (Jeffries *et al.*, 2004; Proikas-Cezanne *et al.*, 2007). To investigate whether *Pf*MyosinF is associated to PI3P positive structures, the PI3P-marker P40PX was used to assess the relative localization of *Pf*MyosinF and PI3P positive structures (section 4.3.5). Since PI3P is a hallmark of early endosomes in eukaryotic cells (Balla, 2013) and was detected earlier at vesicles involved in host cell cytosol uptake (Jonscher *et al.*, 2019), this would give further support for a function of *Pf*MyosinF in the endolysosomal pathway. Additionally, it was shown in *Toxoplasma* that *Tg*MyosinF is involved in the organization of vesicle transport and of the endomembrane system (Carmeille *et al.*, 2021). Inactivation of *Pf*MyosinF in this thesis via knock sideways demonstrated that the mislocalization of the protein into another compartment increased the localization of PI3P-positive structures (represented by the P40PX-marker) at the FV in trophozoites (section 4.3.5). It is important to mention that only one experiment in an asynchronous culture was performed to assess this association, and further that *Pf*MyosinF is refractory to complete mislocalization and thus some of this protein remained at the FV. Nevertheless, this experiment, as well as the bloated food vacuole assay, suggest that

*Pf*MyosinF has a function in hemoglobin endocytosis. For more definite results, these experiments need to be repeated with more parasites, and additionally in a further step, synchronized cultures could be used to better isolate the detected effect.

As the data presented here showed that *Pf*MyosinF is located close to *Pf*Kelch13 foci and is additionally involved in hemoglobin uptake, this suggests that *Pf*MyosinF could possibly influence ART resistance. For this, RSAs were performed with parasites wherein *Pf*MyosinF was inactivated prior to the initiation of the RSA (section 4.3.2). This experiment showed that inactivation of *Pf*MyosinF via the knock sideways system did not render parasites resistant to ART, indicating that *Pf*MyosinF is not involved in endocytic processes in ring stages. Two possibilities exist that could explain this result. One option would be that due to the incomplete mislocalization of *Pf*MyosinF an insufficient amount of *Pf*MyosinF is inactivated to assess the effect of ART treatment on the ring stage parasites. A second explanation would be that *Pf*MyosinF is only weakly expressed in early ring stages, the stage which is most important for ART resistance and thus inactivation of the protein in this stage has no effect on ART resistance (section 4.2.1). However, this would also mean that in rings *Pf*MyosinF is not needed for endocytosis, possibly because in this stage endocytic vesicular transport is not actomyosin dependent. This could be tested by measuring endocytosis after inactivation of *Pf*MyosinF in rings using fluorescent tracers loaded into RBC, as done before to show the function of *Pf*Kelch13 in endocytosis in that stage (Birnbaum *et al.*, 2020). To further investigate ART response upon inactivation of *Pf*MyosinF, a trophozoite-stage survival assay (TSA) could be performed, the developmental stage this protein is more strongly expressed.

The knowledge about *Plasmodium* myosins is limited. Six genes encoding myosins have been discovered in the genome of malaria parasites (Wall *et al.*, 2019). Several homologous genes are known in *Toxoplasma* which encode 11 myosin genes in their genome. Initially, the different myosins were named in order of their discovery which was often done independently for each species (Heintzelman & Schwartzman, 1997; Hettmann *et al.*, 2000; Heintzelman & Schwartzman, 2001; Matuschewski *et al.*, 2001; Lew *et al.*, 2002; Heintzelman, 2004; Chaparro-Olaya *et al.*, 2005). Consequently, the problem arose, that non-homologous genes of *Plasmodium* and *Toxoplasma* carried the same name (Chaparro-Olaya *et al.*, 2005). To avoid confusion, a new systematic designation was proposed, leading to similar naming of homologous myosin genes across taxa. This is why *Pf*MyosinF (PF3D7_1329100), which was previously annotated as *Pf*MyosinC, was renamed. The homologue of *Pf*MyosinF in *Toxoplasma* is *Tg*MyosinF. In 2013, *Jacot et al.* showed that *Tg*MyoF is an actin-dependent motor protein that is involved in the correct positioning of the centromers at the beginning of parasite division and is important for the inheritance of the

apicoplast in *Toxoplasma* (Jacot *et al.*, 2013). The data obtained in this thesis for *P. falciparum* indicated that *Pf*MyosinF in some cases localized close to DAPI-stained nuclei in trophozoites and was seen in schizont stage parasites also close to the nuclei. This might indicate a function of *Pf*MyosinF for cell division, but this needs to be further confirmed by other experiments. To assess whether functions in cell division and apicoplast inheritance of *Pf*MyosinF may also be possible in *Plasmodium* parasites, co-localization experiments with an apicoplast marker and centrosome-marker need to be performed.

5.1.3   Other proteins besides *Pf*Kelch13 influence ART resistance

Several proteins of the newly defined Kelch13 compartment were suspected in ART resistance in previous work (Hunt *et al.*, 2007; Hunt *et al.*, 2010a; Borrmann *et al.*, 2013; Henriques *et al.*, 2014; Cerqueira *et al.*, 2017) and were shown to be essential for parasite development in the host red blood cell (Birnbaum *et al.*, 2020). Partial inactivation of *Pf*AP-2μ, *Pf*Eps15, *Pf*UBP1, *Pf*KIC7 rendered parasites resistant to ART, indicating that they are part of the resistance pathway (section 4.4.2). Since it was shown before that non-synonymous SNPs in other genes than *Pf*Kelch13 are able to reduce susceptibility of *P. falciparum* parasites to ART, mutations of different Kelch13 compartment members were tested in the present work for their capacity to cause ART resistance. A special focus was set on non-synonymous mutations that display a low to medium prevalence in Africa, and SNPs were either tested simultaneously in a mutation pool or individually. Creating mutation pools had the advantage to accelerate the screening process since none of the introduced mutations should be harmful for parasite development, as they were found *in vivo*. While a detrimental effect due to combining all mutations cannot be predicted, the fact that most of the pools were successfully integrated (8 of 9), indicates that this was mostly not the case. The only multipool candidate that failed to integrate was *Pf*Eps15, suggesting that the introduced mutations impaired parasite growth. To continue the analysis of several of the *Pf*Eps15 mutations at a time, one possibility would be to subdivide the multipool construct of *Pf*Eps15 into smaller parts for integration and test e.g. N-terminal, mid part, or C-terminal mutation pools separately, leaving the other regions unchanged.

A potentially limiting factor of the mutation pools is the time-consuming cloning process due to the large size of some of the candidate genes. Furthermore, gene synthesis of the mutated recodonized gene sequence is very cost intensive. However, individual testing of each mutation would have been much more labor intensive and in many instances would

also have required the cloning of large fragments. Hence, overall this approach is a viable option to test many mutations with a single cell line.

Testing the different mutation pools in standard RSA revealed that none of the introduced mutations rendered parasites resistant to ART (section 4.5). The mutation pools included data from the MalariaGEN *Plasmodium falciparum* Community Project (MalariaGEN *et al.*, 2021). One future approach for mutation screening could be that the inclusion criteria are adapted in such a way that mutations with medium to higher prevalence are included. Although this may again not reveal African mutations in resistance since non-synonymous SNPs with higher population frequencies would mean that clinical resistance should already have been detected if such mutations would cause ART resistance. Another option would be to test different combinations of mutated genes, based on the hypothesis that different genetic combinations are important for the development of resistance. However, without prior indication for the combination of genes, this is difficult to achieve in a targeted approach due to the large number of possibilities. Overall, taking into consideration that clinical failures with ACTs in Africa seem to be absent or rare, it is at present unclear if any of the Kelch13 compartment proteins can mediate a reduced susceptibility to ART in the field apart from the *Pf*UBP1 mutation already detected (R3138H) that however derives from SEA (Cerqueira *et al.*, 2017).

A further approach to detect SNPs causing ART resistance in this work was attempted by sequencing of field isolate DNA deriving from a Ghanaian patient suffering repeatedly from malaria even after ART treatment. This resulted in several non-synonymous SNPs found in different Kelch13 compartment members which were tested either in the mutation pools or individually. Since no mutation was detected in *Pf*Kelch13, a *Pf*Kelch13-independent cause was suspected to mediate ART resistance in this patient. However, none of the here tested mutations rendered parasites resistant to ART. To finally find the reason for the suspected ART resistance of the parasites from this patient, sequencing of the DNA samples must be continued, including further genes of the Kelch13 compartment. One option would be to sequence the entire genome of these parasites, but without further indicators, the large number of SNPs that can be expected to result from this are unlikely to easily reveal the cause of resistance. Another possibility would be that ART resistance is driven by an altered expression level of a particular candidate. Thus, the respective promotor regions of each candidate could be analyzed for mutations that potentially alter the expression level of the corresponding gene. It should also be noted that resistance of these parasites was not tested *in vitro* as only DNA is available, and it should also be considered that these parasites might have re-surfaced in this patient due to other reason than ART resistance.

Besides the data from different databases and patient sample sequencing, also mutations mentioned in the literature were experimentally tested in this thesis. With this approach two mutations, found in *Pf*AP-2α and *Pf*UBP1, reduced the sensitivity of the parasite to ART (section 4.6). The H817P mutation of *Pf*AP-2α rendered parasites only mildly resistant to ART. This suggests that this protein, which is part of the heterotetrameric adaptor protein complex that co-localizes with the Kelch13 compartment (Birnbaum *et al.*, 2020), may be involved in endocytosis but that the H817P mutation does only minimally affect this process. This would be in line with the only mild resistance phenotype in the parasites this gene was found mutated in and it should also be noted that other changes in these parasites might be involved in the lowered susceptibility to ART (Rocamora *et al.*, 2018). Previous data showed that mutations in the Mu subunit of the AP-2 complex (*Pf*AP-2μ) increased the tolerance of the parasites to ART (Henriques *et al.*, 2015; Henrici *et al.*, 2019) and partial inactivation of this protein also caused resistance as shown in this thesis. To further test the role of the *Pf*AP-2 complex in ART resistance, more mutations in the different *Pf*AP-2 subunits could be tested.

In contrast to the *Pf*AP-2α mutation, introducing the R3138H mutation (Cerqueira *et al.*, 2017) in the *Pf*UBP1 genomic locus resulted in significant resistance of the parasites to ART (section 4.6.4). This goes in line with prior data obtained in murine parasite models where *Pf*UBP1 was associated with ART resistance (Borrmann *et al.*, 2013; Henriques *et al.*, 2014; Henrici *et al.*, 2019).

5.2 *Pf*Kelch13-dependent ART resistance

5.2.1 Further mutations detected in the Kelch13 propeller domain of *Pf*Kelch13 that mediate ART resistance

Molecular markers provide a powerful tool for the molecular surveillance of ART resistance worldwide. This was already shown for earlier resistance monitoring for chloroquine and for sulfadoxine-pyrimethamine resistance, where tracking of the causative genetic markers, *pfcrt* (for CQ), *pfdhfr* and *pfdhps* (for sulfadoxine-pyrimethamine) was performed to adapt treatment policy and prevent treatment failure (Dieckmann & Jung, 1986; Cowman *et al.*, 1988; Fidock *et al.*, 2000; Djimdé *et al.*, 2001; WHO, 2020a). Currently, 11 mutations of *Pf*Kelch13 (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y and A675V) have been validated as resistance conferring mutations, resulting in delayed

parasite clearance (WWARN K13 Genotype-Phenotype Study Group, 2019). The first confirmed marker for ART resistance was C580Y together with Y493H, R539T, and I543T which was firstly detected in SEA along the Thai-Myanmar and Thai-Cambodian borders (Noedl *et al.*, 2008; Dondorp *et al.*, 2009). Molecular surveillance data indicated that resistance emerged independently and spread over the entire SEA subcontinent (Miotto *et al.*, 2015). Outside the GMS, parasites with C580Y mutations were detected and emerged independently in Papua New Guinea and Guyana (Miotto *et al.*, 2020; Mathieu *et al.*, 2020). Various studies assessing the prevalence of African *pfkelch13* mutations have been published and so far, six validated resistance-conferring *pfkelch13* mutations (M476I, P553L, R561H, P574L, C580Y and A675V) have been reported in four different African countries (Kamau *et al.*, 2015; Kayiba *et al.*, 2021; Ndwiga *et al.*, 2021). Recently published work performed with Rwandan samples detected the non-synonymous mutations R561H in *pfkelch13* and validated this mutation as a marker for ART resistance by *in vitro* experiments using CRISPR-Cas9 genome editing and provided evidence for *in vivo* resistance by molecular surveillance (Uwimana *et al.*, 2020; Uwimana *et al.*, 2021). This mutation was already detected in SEA and was listed as validated ART resistance marker (WWARN K13 Genotype-Phenotype Study Group, 2019). Besides Rwanda, the R561H mutation was reported locally in Uganda and Tanzania at low frequency levels (Bwire *et al.*, 2020; Asua *et al.*, 2021; Moser *et al.*, 2021). Interestingly, all *pfkelch13* mutations identified in Africa are present at low frequency but display a high allelic variation (MalariaGEN Plasmodium falciparum Community Project, 2016; Ocan *et al.*, 2019), which may be based on the infrequent use of ART in African countries due to high semi-immunity. In contrast to SEA, where ART was already used in the 1970s for malaria treatment, in Africa ART was implemented between the years 2000 and 2005. Thus, there was less time for the parasite to establish resistance conferring mutations with a high prevalence (Li *et al.*, 1994). Another option for the low frequency of resistance-conferring mutations in Africa may be a genetic background in African *Plasmodium* parasites that is less suitable for ART resistance and *Pf*Kelch13 mutations than the parasites in SEA (Borrmann *et al.*, 2013; Mita *et al.*, 2016; Cerqueira *et al.*, 2017; Demas *et al.*, 2018; Henrici & Sutherland, 2018; Velavan *et al.*, 2019). Besides this, the partner drugs are still very effective in Africa compared to SEA which means that *pfkelch13* mutations cannot establish themselves as the parasites are eliminated by the partner drug.

The non-synonymous *pfkelch13* mutations (V520A, V520I, V589I, and E612K) tested in the present work were present in Africa (V520A, V589I, E612K) and Asia (V520I) with a low prevalence level. While the V520I and the E612K mutation did not render parasites resistant to ART *in vitro*, V520A and V589I conferred moderate resistance to ART (section 4.6.5).

Both mutations are located in the C-terminal Kelch propeller domain (amino acids 443-726). Structural analysis of the *Pf*Kelch13 protein revealed that most allelic variations are located in this propeller domain (Ariey *et al.*, 2014; Ashley *et al.*, 2014; Anderson *et al.*, 2017). The Kelch domain consists of six repeated kelch blades, each blade arranged in a ß-sheet secondary structure (Adams *et al.*, 2000; Ariey *et al.*, 2014). Additionally, the *Plasmodium* Kelch13 protein comprises an *Apicomplexa*-specific N-terminal region and two highly conserved domains, the coiled-coil domain (amino acids 212-341) and the BTB domain (aa 350-437). To date, only two crystal structure analyses (PDB IDs: 4YY8 and 4ZGC) of *Pf*Kelch13 exist which show the conformational arrangement of the BTB and the Kelch propellers (Jiang *et al.*, 2015). Comparative structural and evolutionary analyses published by *Coppee et al.* in 2019 revealed potential functional sites of *Pf*Kelch13 and provided insight in structural alteration caused by the *pfkelch13* mutations C580Y and R539T (Coppée *et al.*, 2019). The bottom side of the *Pf*Kelch13 hexamer contains a shallow pocket which is highly conserved and may provide a binding site for interaction partners (Coppée *et al.*, 2019). These authors noted that resistance conferring mutations are distributed largely across the Kelch domain but are all located outside this shallow pocket. Additionally, they showed that these mutations may lead to a structural destabilization of the Kelch domain. As it is now known that reduced *Pf*Kelch13 levels explain ART resistance (Siddiqui *et al.*, 2017; Yang *et al.*, 2019; Birnbaum *et al.*, 2020), such alterations might mediate ART resistance by regulating protein abundance, stability, or folding. This agrees with data from other studies, demonstrating that binding of the Kelch domain is regulated by the shallow pocket in structurally related Kelch proteins (Canning *et al.*, 2013; Schumacher *et al.*, 2014; Canning *et al.*, 2015). Transferring these findings to the data presented in this thesis suggests that non-synonymous mutations altering the same amino acid position can result in different outcomes due to differential affection on Kelch domain stability. Even though the V520A and the V520I mutation are located at the same position, and both turn valine into an amino acid with hydrophobic properties, only the amino acid change from valine to alanine rendered parasites resistant to ART whereas the change from valine to isoleucine did not (section 4.6.5). This gives a hint that the additional methyl groups present in the V520I mutation may potentially stabilize the shallow pocket or the entire domain and thus protein interactions, while V520A mutation led to a destabilization resulting in resistance. A recently published study by *Goel et al.* also performed a structural analysis of the *Pf*Kelch13 using Small-Angle X-ray Scattering (SAXS), including the N-terminal Apicomplexan-specific domain and the coiled-coil domain, parts that were ignored in prior crystal structure studies (Goel *et al.*, 2021). These authors suggest that the Kelch domain assembles into a hexameric structure, forming a cauldron-like architecture. According to their data, conformational changes due to mutations in the Kelch propeller hexamer cause alterations

affecting the specific associations between the N-terminal ends of the different hexamer stabilizing chains. Consequently, the substrate-specificity of *Pf*Kelch13 might be negatively influenced or protein complex formation may be interfered with, either of which might lead to ART resistance (Goel *et al.*, 2021). However, it should also be noted that no difference in the interaction profile of wild type and *Pf*Kelch13 C580Y was observed, indicating that general stability rather than interaction to a specific substrate is the cause of resistance (Birnbaum *et al.*, 2020).

Altogether, a standardized, prospective surveillance monitoring (which is still lacking in African countries) in combination with molecular modelling of candidate markers would provide information about resistance trends in Africa and could support tailored adaption of treatment policies (Kayiba *et al.*, 2021; Ndwiga *et al.*, 2021).

5.2.2   Does dormancy provide an explanation for ART resistance?

Artemisinin and its derivatives are very short-acting with only a short serum half-life (White, 2008b; Dondorp *et al.*, 2009). It was suggested that this may be the reason for the recrudescence of parasites in patients which occurs with frequency of up to 50% when a three-day ART monotherapy regimen is applied (Bunnag *et al.*, 1991; Looareesuwan *et al.*, 1992; Meshnick *et al.*, 1996; White, 1997; Kyle *et al.*, 1998; Phan *et al.*, 2002). Nevertheless, prolonging ART treatment duration for more than three days did not change the re-emergence of parasites, which indicates that this treatment failure is not based on drug potency or ART pharmacokinetics alone (Nguyen *et al.*, 1993; McIntosh & Olliaro, 2000; Giao *et al.*, 2001; Cheng *et al.*, 2012). Recrudescence after drug treatment has been observed in various organisms such as bacteria, parasitic protozoans, yeast, fungi, and mammalian cells (LaFleur *et al.*, 2006; Sánchez-valdéz *et al.*, 2018; Vallette *et al.*, 2019). For malaria parasites, *in vitro* studies and mathematical modelling led to the proposal of a new hypothesis which suggests that a phenomenon called dormancy is responsible for parasite recrudescence (Kyle, 1996; Hoshen *et al.*, 2000). According to this, a subpopulation of parasites, either resistant to ART or not, enter a dormant status by arrest in the ring phase to evade elimination. This dormant or persistent status was reported in *in vitro* experiments, while *in vivo* data of *P. falciparum* dormancy did not exist to date (Teuscher *et al.*, 2010). However, it was shown in the rodent *P. vinckei* malaria model that ring stage parasite were less susceptible to artesunate treatment than later developmental stages and that the day of treatment more impacts recrudescence than the total artesunate dose that is administered (LaCrue *et al.*, 2011). Thus, they suggest that the rings present

following 24 h artesunate treatment, displaying a condensed nucleus and pyknotic phenotype, are potential dormant *P. vinckei* stages, showing a similar morphology compared to the *in vitro* phenotype (pyknotic parasites) detected in *P. falciparum* after ART treatment (LaCrue *et al.*, 2011). Altogether, the data raised the question whether the phenomenon of dormancy provides a mechanism for ART resistance in *P. falciparum*.

To get more insight into this process (what are the properties of ART surviving parasites on a morphological level?), an ART resistant parasite line carrying the C580Y mutation in the *pfkelch13* locus was imaged by confocal microscopy directly after ART removal during an RSA and additionally 24 h and 39 h post ART removal (section 4.7.1). The confocal imaging revealed that after 39 hours only a small proportion of ART resistant parasites survived the ART pulse, and that the drug treated parasites were less far developed than the control fraction (section 4.7.1). To better define this finding, the average size of the parasites was measured 24 hours after ART removal, demonstrating that the control parasites were significantly larger than the drug treated parasites. Interestingly, the parasites that had the largest average size in the ART treated population were the same size as the smallest parasites measured in the untreated control. Additionally, the "large" parasites belonged to those that were still alive at 39 hours and continued growing. In summary, the data indicates that either ART treatment delays development of resistant parasites in the first part of the asexual cycle, potentially already during drug pulse, or that resistance is based on a subpopulation of parasites that display a generally slower growth. This goes in line with the finding that parasites where *Pf*Kelch13 is inactivated via the knock sideways system have a prolonged ring stage phase and show a reduced level of hemoglobin endocytosis which results in less ART activation (Birnbaum *et al.*, 2020).

These experiments indicated a lower average age of the parasites surviving the ART pulse compared to the untreated control. From this finding two hypotheses are possible that could explain the delayed growth of parasites upon ART treatment: either a reduced growth is induced directly by ART, conferring tolerance to the parasites, or the survivors represent a subpopulation of parasites that would have grown less irrespective of ART treatment but survived due to their lower growth rate. In both cases, the parasites surviving did not take up the morphology associated with dormancy, suggesting that this is not the usual mechanism to overcome ART treatment. A similar conclusion was recently reached in a controlled human infection model (Watts *et al.*, 2020). Recrudescence of *P. falciparum* in parasites with *Pf*Kelch13 mutations is therefore likely not a process akin to what was defined as dormancy (Breglio *et al.*, 2018; Wellems *et al.*, 2020). In line with this, dormancy is not a process that is unique to treatment with ART since it was also reported for parasites

recovering from treatment with mefloquine or pyrimethamine, suggesting it is a more general effect (Nakazawa *et al.*, 2002).

5.2.3 Fitness and *Pf*Kelch13 protein levels vary in parasites with different *pfkelch13* mutations

In this work it was shown that different *pfkelch13* mutations cause different levels of ART resistance (section 4.6.5). Additionally, it was demonstrated that an already resistant cell line became even more resistant by re-growing survivors in consecutive RSAs (section 4.7.2). In 2018, it was published by *Nair et al.* that C580Y and R561H *pfkelch13* mutants differ in their respective fitness level (Nair *et al.*, 2018). This raised the question whether there are differences in fitness level of the C580Y and V520A mutants compared to wild type *Pf*Kelch13 parasites, particularly as the latter mutation resulted in a much lower level of ART resistance. Competitive growth assays revealed that in co-cultivation with 3D7 parasites, the C580Y mutant cell line displayed the highest fitness cost, while the V520A mutation resulted in an intermediate fitness phenotype compared to wild type *pfkelch13* parasites (section 4.8.2). Interestingly, this inversely correlated with the respective parasite survival rate upon ART treatment, showing that highly resistant C580Y parasites had the highest fitness cost whereas the moderately resistant V520A mutants had similar fitness costs than the wild type parasites (section 4.8.2). The data obtained for the C580Y mutants agrees with the data presented by *Nair et al.*, which showed that these mutant parasites have higher fitness costs compared to another *pfkelch13* mutation (R561H) (Nair *et al.*, 2018). These findings also match with observations in bacteria and viruses where it was seen that drug resistant strains displayed reduced fitness compared to sensitive strains (Andersson & Hughes, 2010; Götte, 2012; Wargo & Kurath, 2012). Despite of this, it is quite intriguing that the C580Y mutation is very successful in the field, as it is the most prevalent ART resistance mutation in SEA (e.g. ~ 88% in Cambodia, WWARN). In contrast, the V520A mutation is present in African countries at rather low frequencies (e.g. ~1.3% in Ghana and ~4% in DRC, WWARN) and is absent in SEA. Since this mutation only caused a very mild fitness reduction (section 4.8.2), this could be beneficial in African settings where infections with multiple *P. falciparum* parasite strains is common in high-transmission areas (Arnot, 1998; Juliano *et al.*, 2010). This multiplicity of infection causes intra-host competition between different strains, resulting in an increased negative selection of the parasite strain suffering the largest fitness cost (Roode *et al.*, 2003; Roode *et al.*, 2004). Thus, it may be advantageous for parasites in African settings to display only mild ART resistance, resulting

in lower fitness cost, to be able to survive in a polyclonal host environment. Additionally, in malaria endemic areas with a high parasite transmission rate, an overall stable anti-disease immunity exists against *P. falciparum* infections, resulting in less frequent drug treatment (Bull & Marsh, 2002; Rosenthal, 2013; Fowkes *et al.*, 2016). In contrast, mutations rendering parasites resistant to ART at a high level - such as C580Y - may have an advantage to survive among other parasites strains if treatment is frequent.

Another factor that may influence the fitness of ART resistant parasites are additional genomic adaptions to compensate growth disadvantages in the absence of selection pressure, which are due to the resistance mechanism (Walliker *et al.*, 2005). For this, the parasite needs to establish compensatory genetic modifications to be able to persist besides the wild type parasites, which is energetically cost-intensive and consequently results in a lowered fitness level. The importance of the genetic background was highlighted by work showing that the introduction of C580Y in different field isolates deriving either from SEA, or Africa, resulted in different fitness cost but also different levels of resistance (Straimer *et al.*, 2017; Nair *et al.*, 2018; Stokes *et al.*, 2021). For future experiments, it would be interesting to introduce the V520A mutations into field isolates deriving from Africa to assess the difference in fitness and resistance in different genetic backgrounds. Further research is also needed to define the impact of secondary genetic factors on parasite fitness that mediate ART resistance in a *Pf*Kelch13-independent manner (e.g *Pf*UBP1, *Pf*coronin, *Pf*AP-2μ) (Henriques *et al.*, 2014; Cerqueira *et al.*, 2017; Demas *et al.*, 2018; Henrici *et al.*, 2019). Mutations in *pfkelch13* often render parasites resistant to ART to a higher extent than mutations in *pfubp1* or *pfap-2μ*. As *Pf*Kelch13 function is only relevant in ring stages but *Pf*UBP1 or *Pf*AP-2μ are important in all asexual developmental stages, the latter mutations might result in a higher fitness cost. To investigate this idea, competition assays could be performed with parasites carrying a mutation in *pfkelch13* versus parasites carrying for instance a *pfubp1* mutation.

Besides looking at the fitness phenotype of different resistance conferring mutations, one can also speculate about the mechanisms behind the differences in fitness cost. *Birnbaum and Scharf et al*. demonstrated that ART resistance is based on a reduced hemoglobin endocytosis which results in less activated ART (Birnbaum *et al.*, 2020). Additionally, it was shown that ART resistant parasites showed lower abundance of *Pf*Kelch13, indicating that *Pf*Kelch13 protein levels and levels of endocytosis correlate with resistance. Based on this finding, the *Pf*Kelch13 protein levels of different *Pf*Kelch13 mutants were measured in this work. Fluorescence intensity measurements revealed, that the C580Y and the highly resistant C580Y mutant obtained by consecutive RSAs (K13[C580Y_29th]) had the lowest *Pf*Kelch13 protein level compared to *Pf*Kelch13 wild type parasite, while the V520A mutant

showed an intermediate *Pf*Kelch13 protein level (section 4.8.1). This data, in combination with the data published by *Birnbaum and Scharf et al.*, indicates that due to lower *Pf*Kelch13 protein amount the C580Y mutants show a reduced hemoglobin endocytosis, reducing the fitness level of these mutants. In contrast, the V520A mutants have lower fitness costs due to a more moderate reduction in *Pf*Kelch13 protein levels but likely also more moderate downregulation of hemoglobin uptake and, as demonstrated here, more moderate resistance. To gain further insight into this correlation, hemoglobin uptake assays need to be performed with the different *pfkelch13* mutants.

6   References

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List of publications

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Appendix

Appendix A

Appendix A.1 Determination of relative position of MCA2^{wt}-3xHA and K13.

Relative localization MCA2/K13	Date	Image	MCA2 foci	K13 foci	Co-loc	partial co-loc	no co-loc
	02.12.2020	DAPI61ed	1	1	1	0	0
		DAPI60ed	1	2	0	1	0
		DAPI60ed	1	1	0	1	0
		DAPI59ed	1	2	0	0	1
		DAPI58ed2	1	2	0	0	1
		DAPI58ed	1	4	1	0	0
		DAPI57ed	1	1	1	0	0
		DAPI57ed	2	5	1	0	1
		DAPI56ed	1	5	1	0	0
	04.12.2020	DAPI59ed4	2	2	1	0	1
		DAPI59ed3	1	2	0	1	0
		DAPI59ed	6	3	0	2	4
		DAPI58ed2	2	4	2	0	0
		DAPI58ed	1	1	0	1	0
		DAPI57ed	3	3	2	0	1
		DAPI56ed2	2	4	0	1	1
		DAPI65ed	3	3	0	1	2
		DAPI64ed	3	6	1	1	1
		DAPI63ed	2	3	1	0	1
		DAPI62ed3	2	2	2	0	0
		DAPI62ed2	1	4	1	0	0
		DAPI62ed	1	3	1	0	0
		DAPI73ed	2	2	2	0	0
		DAPI73ed	1	2	1	0	0
		DAPI72ed	1	4	1	0	0
		DAPI70ed	1	1	0	0	1
		DAPI69ed	2	2	1	1	0
		DAPI68ed2	1	3	1	0	0
		DAPI68ed	2	3	0	2	0
		DAPI66ed	1	1	0	0	1
Summe		30	50	81	22	12	16
Proportion					44	24	32

Appendix A.2 Determination of relative position of MCA2^{Y1344Stop}-GFP and K13.

Relative location MCA2/K13 (Tropfen)	Date	Image	MCA2 (Y1344.)	Kelch13	Co-loc	partial co-loc	no co-loc
	29.05.2020	Alexa09ed2	2	2	1	1	0
	12.06.2020	Alexa10ed	3	6	2	1	0
		Alexa11ed	3	4	3	0	0
		Alexa12ed	2	1	1	0	1
		GFP07ed	2	3	1	1	0
		GFP07ed2	5	6	4	0	1
	01.07.2020	Alexa04ed	1	2	0	0	1
		Alexa04ed2	2	2	1	0	1
		Alexa09ed	2	1	1	0	1
		Alexa12ed	1	1	0	1	0
		Alexa14ed	2	2	2	0	0
		Alexa20ed3	1	2	1	0	0
		Alexa31ed	1	1	1	0	0
		Alexa31ed2	2	2	1	1	0
		Alexa32ed	2	1	1	0	1
		GFP05ed	1	1	1	0	0
		GFP07ed	1	1	1	0	0
		GFP07ed2	2	3	2	0	0
		GFP11ed2	1	1	1	0	0
		GFP11ed2	1	1	1	0	0
		GFP11ed2	1	1	1	0	0
		GFP11ed2	1	1	1	0	0
		GFP12ed2	2	2	0	2	0
		GFP14ed	1	1	0	1	0
		GFP16ed	1	1	1	0	0
		GFP18ed	1	1	1	0	0
		GFP18ed2	1	1	1	0	0
		GFP19ed	1	1	0	0	1
		GFP22ed2	1	1	0	0	1
		GFP23ed	1	1	1	0	0
		GFP24ed2	2	1	0	0	2
		GFP25ed	1	1	1	0	0
		GFP25ed3	3	2	0	2	1
		GFP29ed2	1	1	1	0	0
		GFP29ed4	1	1	0	1	0
		GFP30ed2	1	1	0	0	1
		GFP30ed3	3	3	3	0	0
		GFP35ed	1	1	1	0	0
		GFP35ed2	1	1	0	1	0
	17.08.2020	Alexa06ed	1	1	1	0	0
		GFP10ed	1	1	1	0	0
		GFP10ed2	1	1	0	1	0
		GFP11ed	1	1	0	0	1
		GFP12ed	3	3	2	1	0
		GFP12ed2	1	1	0	0	1
		GFP13ed	1	1	0	0	1
Summe		n=46	71	75	42	14	15
Co-loc MCA2/K13					59		
Partial Co-loc						20	
no co-loc							21

Appendix A.3 Determination of relative position of MCA2^{Y1344Stop} and K13 in smears.

Relative location MCA2/K13 (Smear)	Date	Image	MCA2 (Y1344.)	Kelch13	Co-loc	partial co-loc	no co-loc
	27.07.2020	GFP76ed	2	1	1	0	1
		GFP79ed	2	1	1	0	1
		GFP79ed	2	2	2	0	0
		GFP79ed2	1	1	1	0	0
		GFP81ed	1	1	1	0	0
		GFP82ed2	2	2	2	0	0
		GFP83ed	1	2	1	0	0
		GFP86ed	1	1	1	0	0
		GFP89ed2	2	1	1	0	1
		GFP93ed	1	2	1	0	0
		GFP94ed	2	1	1	0	1
		GFP94ed	1	1	1	0	0
	17.08.2020	GFP17ed	1	1	1	0	0
		GFP18ed	2	1	1	0	1
		GFP19ed	3	1	1	0	2
		GFP19ed2	2	2	1	0	1
		GFP20ed	3	2	2	0	1
		GFP22ed	3	3	3	0	0
		GFP25ed	2	1	1	0	1
		GFP27ed	2	2	2	0	0
Summe	n=20		36	29	26	0	10
Co-loc MCA2/K13					72		
Partial Co-loc						0	
no co-loc							28

Appendix B

Appendix B.1 Determination of relative position of MyoF^{wt}-3xHA and K13 (acetone IFA).

MyosinF (3xHA) + K13mCh				mCherry	GFP					
ACETON										
	Date	Image	Stage	# of MyoF foci	# of Kelch foci	# co-loc	# partial co-loc	# close foci	no co-loc	remarks
	Aceton IFA (vom 26.06.2020)	DAPI19ed2	R	2	5	1	0	1	0	no hemozoine
		DAPI19ed3	R	1	1	0	0	1	0	no hemozoine
		DAPI19ed4	T	1	2	0	0	1	0	Kelch foci close to hemozoine but not MyoF
		DAPI19ed6	R	2	2	1	0	1	0	no hemozoine
		DAPI19ed7	R	3	1	0	1	2	0	no hemozoine
		DAPI19ed8 (1)	R	2	3	0	1	1	0	no hemozoine
		DAPI19ed8 (2)	R	1	3	0	0	1	0	no hemozoine
		DAPI19ed10 (1)	T	1	2	0	0	1	0	Kelch foci close to hemozoine but not MyoF
		DAPI19ed10 (2)	R	1	2	0	0	1	0	no hemozoine
		DAPI19ed11	T	2	3	1	0	1	0	1 MyoF focus close to FV
		DAPI20ed2	R	3	2	1	0	2	0	no hemozoine
		DAPI20ed3	R	1	1	0	0	1	0	no hemozoine
		DAPI21ed	R	1	3	0	0	1	0	no hemozoine
		DAPI21ed2	R	2	1	0	0	2	0	no hemozoine
		DAPI21ed3	R	1	2	0	1	0	0	no hemozoine
		DAPI21ed5	R	2	1	0	0	2	0	no hemozoine
		DAPI22ed2	R	1	1	0	1	0	0	no hemozoine
		DAPI22ed3	R	5	4	3	1	1	0	no hemozoine
		DAPI23ed	T	1	2	0	1	0	0	no hemozoine
		DAPI23ed2	R	2	1	0	0	2	0	no hemozoine
		DAPI23ed5	T	3	1	0	0	3	0	1 MyoF focus close to FV
		DAPI24ed	T	3	2	0	0	2	1	2 MyoF foci close to FV
		DAPI25ed4	T	2	2	0	0	2	0	2 MyoF foci close to FV
		DAPI25ed5	T	2	3	0	1	1	0	1 MyoF focus close to FV
		DAPI25ed7	R	1	1	0	0	1	0	no hemozoine
		DAPI25ed8	R	3	1	0	1	2	0	no hemozoine
		DAPI25ed11	T	1	2	1	0	0	0	1 MyoF focus close to FV
		DAPI26ed (1)	T	2	3	0	2	0	0	2 MyoF foci close to FV
		DAPI26ed (2)	T	2	3	0	1	1	0	1 MyoF focus close to FV
		DAPI26ed2	R	1	1	0	0	1	0	no hemozoine
		DAPI26ed3	R	2	2	1	0	1	0	no hemozoine
SUMME			31	57	63	9	11	36	1	
Co-loc MyoF(3xHA)/K13						16				
Partial co-loc MyoF(3xHA)/K13							19			
Close foci MyoF(3xHA)/K13								63		
No co-loc MyoF(3xHA)/K13									2	
Co-loc MyoF/K13 at FV						2				
MyoF foci at FV										11
Co-loc at FV [%]		18								

Appendix B.2 Determination of relative position of MyoF^{wt}-3xHA and K13 (formaldehyde/glutaraldehyde IFA).

MyosinF (3xHA) + K13mCh
FORMALDEHYDE/GLUTHARALDEHYDE

	Date	Image	Stage	mCherry # of MyoF foci	GFP # of Kelch foci	# co-loc	# partial co-loc	# close foci	no co-loc	remarks
	In solution IFA (vom 26.06.2020)	DAPI51ed	R	3	1	1	0	0	2	no hemozoine
		DAPI54ed2	R	3	1	0	0	0	3	no hemozoine
		DAPI57ed	T	1	4	0	0	1	0	1 MyoF focus close to FV
		DAPI58ed	T	2	4	0	1	1	0	MyoF apart of FV at cell periphery
		DAPI59ed	T	2	6	1	1	0	0	1 MyoF focus close to FV
		DAPI59ed2	R	4	1	1	0	0	3	no hemozoine
		DAPI62ed	T	1	3	0	1	0	0	1 MyoF focus close to FV
		DAPI63ed	T	1	6	1	0	0	0	1 MyoF focus close to FV
		GFP26ed	T	5	6	0	0	4	1	3 MyoF foci close FV
		GFP26ed2	T	7	5	1	0	4	2	3 MyoF foci close FV
SUMME			**10**	29	37	5	3	10	11	
Co-loc MyoF(3xHA)/K13						17				
Partial co-loc MyoF(3xHA)/K13							10			
Close foci MyoF(3xHA)/K13								34		
No co-loc MyoF(3xHA)/K13									38	
MyoF in trophs										7
MyoF in trophs at FV										6
MyoF near FV [%]										86
Co-loc MyoF/K13 in rings [%]						20				
Co-loc MyoF/K13 in trophs [%]						16				
Co-loc MyoF/K13 in trophs at FV [%]						30				

Appendix B.3 Determination of relative position of MyoF^{wt}-2x2 and K13.

MyosinF (2x2FKBP) + K13mCh

	Date	Image	Stage	GFP # of MyoF foci	mCherry # of Kelch foci	# co-loc	# partial co-loc	# close foci	no co-loc	remarks
	16.11.2020 (63x)	Alexa11ed	ET	2	1	0	2	0	0	2 MyoF foci close to FV
		Alexa11ed3	LT	2	1	0	0	0	2	2 MyoF foci close to FV
		Alexa11ed4	ER	1	2	0	0	0	1	no hemozoine
		Alexa12ed	LT	2	1	0	0	0	2	2 MyoF foci close to FV
		Alexa12ed2	ET	3	1	0	0	2	1	3 MyoF foci close to FV
		GFP54ed	LT	1	2	0	0	1	0	1 MyoF focus close to FV
		GFP55ed	ET	1	1	0	0	1	0	MyoF apart of FV
	16.11.2020 (100x)	GFP60ed	ET	1	1	1	0	0	0	MyoF apart of FV
		GFP61ed	LR	1	1	0	0	0	1	1 MyoF focus close to FV
		GFP62ed	LT	2	1	0	0	2	0	2 MyoF foci close to FV
	02.10.2020	Alexa07ed	ET	4	2	0	1	1	2	2 MyoF foci close to FV
	23.11.2020	GFP77ed	LT	1	1	0	0	0	1	MyoF apart of FV
		GFP81ed	LT	2	3	0	0	2	0	MyoF apart of FV
		GFP82ed	LT	2	1	1	0	0	1	1 MyoF focus close to FV
SUMME			**14**	25	19	2	3	9	11	
Co-loc MyoF(2x2)/K13						8				
Partial co-loc MyoF(2x2)/K13							12			
Close foci MyoF(2x2)/K13								36		
No co-loc MyoF(2x2)/K13									44	
Total MyoF in late ring and trophs										24
MyoF in late ring and trophs at FV										16
MyoF near FV [%]										67
Co-loc MyoF/K13 in late rings and trophs at FV [%]						13				

Appendix B.4 Independent replications of bloated food vacuole assay performed with MyoF^{wt}-2x2+1xNLS.
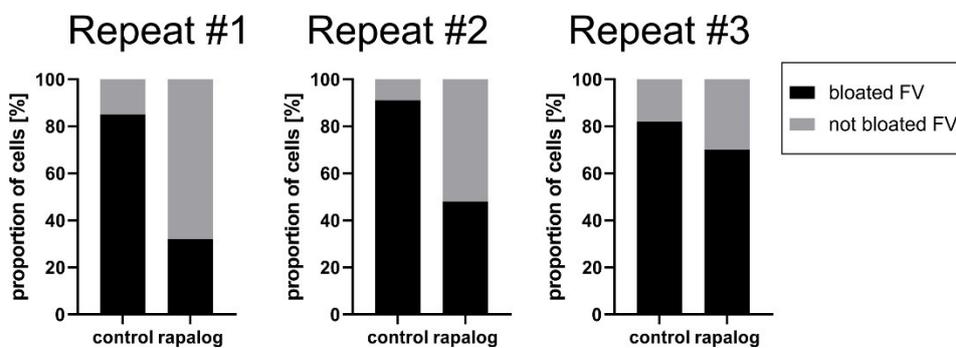
Appendix B.5 Assessment of relative position of MyoF^{wt}-2x2 and K13 in the presence of 10 µM Cytochalasin D at two different time points. *green* (ET) early trophozoite; *orange* (LT), late trophozoite; *blue* (ER), early ring (rings were excluded from calculations).

Zeitpunkt	Bild	Stadium	Foci K13	Foci MyosinF	Co-loc	Partial co-loc	No co-loc
Start	GFP21ed	LT	2	4	1	0	3
	GFP21ed2	ET	1	1	0	0	1
	GFP18ed	LT	1	4	1	0	3
	GFP17ed	LT	2	1	0	0	1
	GFP15ed	ET	2	2	1	0	1
	GFP12ed2	ET	1	1	0	0	1
	GFP12ed	ET	1	1	0	0	1
	GFP11ed3	ET	1	1	0	0	1
	GFP11ed	LT	1	3	1	0	2
	GFP09ed	ET	1	2	1	0	1
	GFP15ed2	ET	1	1	0	1	0
	GFP15ed3	ET	1	1	0	0	1
	GFP16ed	ET	1	1	0	0	1
	GFP13ed3	ET	1	1	0	1	0
	GFP13ed3	LT	1	1	0	0	1
	GFP19ed2	ET	1	3	0	0	3
	GFP17ed2	ET	2	3	1	0	2
Summe	**n=17 cells**						
Summe foci (ET)			14	18	3	2	13
Co-loc MyoF(2x2)/K13					17		
Partial co-loc MyoF(2x2)/K13						11	
No co-loc MyoF(2x2)/K13							72
Summe foci (LT)			7	13	3	0	10
Co-loc MyoF (2x2)					23		
Partial co-loc MyoF (2x2)						0	
No co-loc MyoF (2x2)							77

Zeitpunkt	Bild	Stadium	Foci K13	Foci MyosinF	Co-loc	Partial co-loc	No co-loc
Kontrolle	GFP54ed	ET	1	2	1	0	1
1h nach Start	GFP53ed	LT	1	3	1	0	2
	GFP52ed	LT	1	2	0	0	2
	GFP51ed	ET	1	1	0	0	1
	GFP48ed	ET	1	1	0	0	1
	GFP47ed	ET	1	2	1	0	1
	GFP46ed	ET	1	3	0	0	3
	GFP44ed	ET	2	1	0	1	0
	GFP43ed	ET	1	4	1	0	3
	GFP42ed	LT	1	2	1	0	1
	GFP50ed2	ET	1	2	1	0	1
	GFP50ed	ET	1	1	0	0	1
	GFP49ed	LT	2	3	1	0	2
Summe	**n=13 cells**						
Summe foci (ET)			10	17	4	1	12
Co-loc MyoF(2x2)/K13					24		
Partial co-loc MyoF(2x2)/K13						6	
No co-loc MyoF(2x2)/K13							71
Summe foci (LT)			5	10	3	0	7
Co-loc MyoF (2x2)					30		
Partial co-loc MyoF (2x2)						0	
No co-loc MyoF (2x2)							70

Zeitpunkt	Bild	Stadium	Foci K13	Foci MyosinF	Co-loc	Partial co-loc	No co-loc
mit CytD (10µM)	GFP41ed	LT	1	3	0	1	2
1h nach Start	GFP40ed2	LT	5	8	3	0	5
	GFP39ed	LT	1	4	1	0	3
	GFP39ed2	LT	1	2	0	1	1
	GFP39ed3	LT	2	2	1	0	1
	GFP37ed	ET	1	1	0	0	1
	GFP36ed	ET	1	2	0	0	2
	GFP33ed2	ET	1	1	0	1	0
	GFP30ed	LT	1	2	1	0	1
	GFP28ed	LT	1	1	0	1	0
	GFP27ed	ET	1	1	0	1	0
	GFP26ed	LT	4	5	2	0	3
	GFP25ed	LT	2	1	0	0	1
	GFP24ed	LT	1	1	0	0	1
	GFP22ed (1)	LT	1	2	0	0	2
	GFP22ed (2)	ET	2	2	0	0	2
	GFP31ed2 (1)	LT	1	2	0	0	2
	GFP31ed2 (2)	LT	1	1	0	0	1
	GFP31ed	LT	1	2	1	0	1
	GFP40ed3	ER	1	1	0	1	0
Summe	**n=20 cells**						
Summe foci (ET)			6	7	0	2	5
Co-loc MyoF(2x2)/K13					0		
Partial co-loc MyoF(2x2)/K13						29	
No co-loc MyoF(2x2)/K13							71
Summe foci (LT)			23	36	9	3	24
Co-loc MyoF (2x2)					25		
Partial co-loc MyoF (2x2)						8	
No co-loc MyoF (2x2)							67

Appendix B.6 Assessment of relative position of MyoF^wt-2x2 and P40PX in the presence of rapalog. at four different time points. Color code represents different time points. *FV*, food vacuole; *V*, vesicles

	Timepoint	Treatment	Image	MyoF (FV)	MyoF (V)	Co-Loc (FV)	Co-loc (V)	close foci (FV)	close foci (V)	no co-loc
	Start	control	Alexa17ed	1	0	0	0	1	0	0
			Alexa16ed	1	0	0	0	1	0	0
			GFP04ed	1	0	0	0	0	1	0
			Alexa13ed	2	0	0	0	2	0	0
			Alexa06ed	1	0	1	0	0	0	0
			Alexa10ed	2	0	0	0	2	0	0
			Alexa12ed2	2	0	2	0	0	0	0
	2 h	control	Alexa36ed	1	0	1	0	0	0	0
			Alexa35ed2	2	0	0	0	1	1	0
			Alexa34ed	1	0	0	1	0	0	0
			Alexa33ed	0	1	0	0	0	1	0
			Alexa32ed	2	1	0	0	2	0	1
			Alexa30ed	3	0	0	0	3	0	0
		rapalog	Alexa29ed	1	1	1	1	0	0	0
			Alexa28ed	1	0	1	0	0	0	0
			Alexa27ed	1	0	1	0	0	0	0
			Alexa26ed	1	2	1	1	0	1	0
			Alexa24ed	2	0	2	0	0	0	0
			Alexa22ed	1	0	1	0	0	0	0
			Alexa21ed	1	0	1	0	0	0	0
			Alexa18ed	1	0	1	0	0	0	0
			Alexa23ed	2	0	2	0	0	0	0
			Alexa25ed	2	0	2	0	0	0	0
			Alexa26ed2	2	0	2	0	0	0	0
			Alexa28ed2	1	1	1	0	0	1	0
			Alexa20ed	2	0	2	0	0	0	0
	4 h	control	Alexa57ed	2	0	2	0	0	0	0
			Alexa56ed	2	0	0	0	2	0	0
			Alexa54ed	2	0	2	0	0	0	0
			Alexa53ed	2	0	1	0	1	0	0
		rapalog	Alexa52ed	1	0	1	0	0	0	0
			Alexa51ed	1	0	1	0	0	0	0
			Alexa50ed	1	0	1	0	0	0	0
			Alexa49ed	1	1	1	1	0	0	0
			Alexa47ed	1	0	1	0	0	0	0
			Alexa46ed2	1	0	1	0	0	0	0
			Alexa44ed	2	0	1	0	1	0	0
			Alexa42ed	1	0	1	0	0	0	0
			Alexa41ed	1	0	0	0	1	0	0
			Alexa45ed	1	1	1	1	0	0	0
			Alexa46ed	1	0	1	0	0	0	0
	24 h	control	Alexa25ed2	2	0	1	0	1	0	0
			Alexa24ed	1	0	1	0	0	0	0
			Alexa22ed	1	1	1	0	0	1	0
			Alexa21ed	1	0	0	0	1	0	0
			Alexa20ed	1	0	1	0	0	0	0
			Alexa19ed	1	0	1	0	0	0	0
			Alexa18ed	2	0	0	0	0	0	2
			Alexa15ed	1	0	1	0	0	0	0
			Alexa15ed2	2	0	2	0	0	0	0
			Alexa17ed	1	0	1	0	0	0	0
			Alexa25ed	2	0	2	0	0	0	0
		rapalog	Alexa13ed	2	0	2	0	0	0	0
			ALexa11ed	2	0	2	0	0	0	0
			Alexa10ed	1	0	1	0	0	0	0
			Alexa08ed	1	1	1	1	0	0	0
			Alexa07ed	1	0	1	0	0	0	0
			Alexa06ed	1	0	0	0	1	0	0
			Alexa14ed	2	0	2	0	0	0	0

	Timepoint	Treatment	Image	MyoF (FV)	MyoF (V)	Co-Loc (FV)	Co-loc (V)	close foci (FV)	close foci (V)	no co-loc (FV)	no co-loc (V)
Summe	Start	control	n=7	10	0	3	0	6	1	0	
	2 h	control	n=6	9	2	1	1	6	2	1	
		rapalog	n=13	18	4	18	2	0	2	0	
	4 h	control	n=4	8	0	5	0	3	0	0	
		rapalog	n=11	12	2	10	2	2	0	0	
	24 h	control	n=11	15	1	11	0	2	1	2	
		rapalog	n=7	10	1	9	1	1	0	0	

	Timepoint	Treatment	Image	MyoF (gesamt)	Co-Loc (gesamt)	close foci (gesamt)	no co-loc
Summe	Start	control		10	3	7	
Gesamtauswertung	2 h	control		11	2	8	
		rapalog		22	20	2	
	4 h	control		8	5	3	
		rapalog		14	12	2	
	24 h	control		16	11	3	
		rapalog		11	10	1	
Prozentualer Anteil	Start	control			30	70	0
Gesamtauswertung	2 h	control			18	73	9
		rapalog			91	9	0
	4 h	control			63	38	0
		rapalog			86	14	0
	24 h	control			69	19	13
		rapalog			91	9	0

Appendix C

Primers used for cloning and sequencing of DNA samples from Ghanain field isolate

#	Oligo Name	Sequence 5` to 3`
1	1 Kelch13 (PF3D7_1343700) fwd	GGCGTAAATATTCGTGTTATAATTTCTCCAAG
2	1 Kelch13 (PF3D7_1343700) rev	GTGCATGAAAATAAATATTAAAGAAG
3	2 Formin2 (PF3D7_1025000) fwd	GAAAAAGATATATACTAAATATTTCTACAC
4	2 Formin2 (PF3D7_1025000) rev	CACCATTTTTATCATCATTTTCTTCGTCGTCTCC
5	3 Formin2 (PF3D7_1025000) fwd	GCATCTAGGAGAAGAGGAAGAAGAAATGAAAAG
6	3 Formin2 (PF3D7_1025000) rev	CAAACAAAGGAATATAAAATAATATATAGGG
7	4 UBP1 (PF3D7_0104300) fwd	CGAAAGAAGAAAATAATAATAATTAATAAAAGG
8	4 UBP1 (PF3D7_0104300) rev	GGTATATTATTACTTTCTATAATTTTCATATCACTACTAC
9	5 UBP1 (PF3D7_0104300) fwd	CAATGATAATAATATGAACAGTAATAATAACAAC
10	5 UBP1 (PF3D7_0104300) rev	CATAATTGTTTTTGTTATCATCATCTTTTTGG
11	6 UBP1 (PF3D7_0104300) fwd	GGGTTCACCAAGAAATAGTATAAAAAGGGAGG
12	6 UBP1 (PF3D7_0104300) rev	CTATTGGATATATTACTATCATCGTCACTATC
13	7 UBP1 (PF3D7_0104300) fwd	GATGATAATTTAGGAAATAATATATTCCATCC
14	7 UBP1 (PF3D7_0104300) rev	GTTTAATTTTTGTTCGCTATTCTTG
15	8 MyosinC (PF3D7_1329100) fwd	TTTTATTTAATATATAAACAAATTGTAGGGCG
16	8 MyosinC (PF3D7_1329100) rev	CCTCATTCATTGTTGTGGTTGTATCTTGC
17	9 MyosinC (PF3D7_1329100) fwd	GGCCAATTTTATACTCTCCAGTTTATATGTAAC
18	9 MyosinC (PF3D7_1329100) rev	GTTCACTGTGTTGATTAGGAGGTACAGAGG
19	10 MyosinC (PF3D7_1329100) fwd	GATATATATGATATAGTAAATAATTATGC
20	10 MyosinC (PF3D7_1329100) rev	CATAGGAATATATTCAAATGTATACAC
21	11 KBI9 (PF3D7_0813000) fwd	GGAAAAAAGAAAGAAGAAATTGTACATAATAAATAAG
22	11 KBI9 (PF3D7_0813000) rev	GATACACACATGATATCATACATAAGGC
23	12 Unknown (PF3D7_0609700) fwd	CCATTTTATTAAAACAAACCTTAGATAC
24	12 Unknown (PF3D7_0609700) rev	CTTTCATGTTTGTTCCTTTTCCTTTCCCG
25	13 Unknown (PF3D7_0609700) fwd	GTTAATTTTGATGAAATAAATCCTAATGG
26	13 Unknown (PF3D7_0609700) rev	GTAATAATTTCGTATTTACTACAATTCGAC
27	14 Unknown (PF3D7_0609700) fwd	CGAATGTAAGGAATATAACCATATG
28	14 Unknown (PF3D7_0609700) rev	GGTGAAAAATATAGAAAATCTAATATTCG

33	1a Kelch13 (PF3D7_1343700) fwd	CCATTGATATGAGTGTATTAGATTCGAAC
34	1b Kelch13 (PF3D7_1343700) fwd	CGGAATTAAGTGATGCTAGTGATTTTG
35	1c Kelch13 (PF3D7_1343700) fwd	GAGAATGATAAAAAAAAAATTGTTGATGC
36	1d Kelch13 (PF3D7_1343700) fwd	CGATGGAATTATTAGATATTAGTCAAC
37	1e Kelch13 (PF3D7_1343700) fwd	GGGGGATATGATGGCTCTTCTATTATACCG
38	1f Kelch13 (PF3D7_1343700) fwd	CATATTAGATTCCGTTGAACAATATCAACC
39	2a Formin2 (PF3D7_1025000) fwd	CGGCCTCTTCCTTTCTTCAGAATTCGGG
40	2b Formin2 (PF3D7_1025000) fwd	GTTGTCGTCTTGTTGCTCATGCTCAGAATGG
41	2c Formin2 (PF3D7_1025000) fwd	CCCGCTTGTTTACCCAGCTTCGATATTGTTAG
42	2d Formin2 (PF3D7_1025000) fwd	CCCTCTTTCTGTACCCATGGAGTTGTTAC
43	3a Formin2 (PF3D7_1025000) fwd	CGTCAAGGACATGTTGTGGATAAGGATTTA
44	3b Formin2 (PF3D7_1025000) fwd	GAAGATGCACGGTAAGGGTGATGATG
45	3c Formin2 (PF3D7_1025000) fwd	GAAGAGATAAATAGAAAACTTGAAGAAGAAAT
46	3d Formin2 (PF3D7_1025000) fwd	GATGAAAAAAATCTTATCATTTTGAAAAATATG
47	3e Formin2 (PF3D7_1025000) fwd	GCCTATCGACAATTATGTTTTGTGTAACGC
48	3f Formin2 (PF3D7_1025000) fwd	GGTGATAGCGTAGATATTTTTTCAAGCCTTG
49	4a UBP1 (PF3D7_0104300) fwd	CGACTCGTTCAGCTTATCTAATACG
50	4b UBP1 (PF3D7_0104300) fwd	CCATAGCGATAGTATTAATAATAGTATTAA
51	4c UBP1 (PF3D7_0104300) fwd	GATGATAATATGGATGACGATGATGATG
52	4d UBP1 (PF3D7_0104300) fwd	GATACCATAGATGATGTTTTTAAAAATAAAAG
53	4e UBP1 (PF3D7_0104300) fwd	CATCAAATAGTATGTATAAAGATTATTCG
54	4f UBP1 (PF3D7_0104300) fwd	GCACATGAGGGGTTGTCAAAAAAAATGTTG
55	4g UBP1 (PF3D7_0104300) fwd	GATGATATGGAAGAAATGACAAGATTTAG
56	5a UBP1 (PF3D7_0104300) fwd	GGTCTCTTCATGAAGATCTTTTAAAAGAAG
57	5b UBP1 (PF3D7_0104300) fwd	CATCTTAATAATCATGAGAGTGATGAC
58	5c UBP1 (PF3D7_0104300) fwd	GAACGATTCAGGTGATTTTGCGGTGG
59	5d UBP1 (PF3D7_0104300) fwd	CGCCTTTTACACAATAAACATTTTAAAG
60	5e UBP1 (PF3D7_0104300) fwd	CGAAAAATATGACAAATATGACAAATATG
61	5f UBP1 (PF3D7_0104300) fwd	GAGTGTTTTGATAGAAGAAAATAATAGTATG
62	5g UBP1 (PF3D7_0104300) fwd	GGTGGTGATAAGAATCGTCGTAATAATTTT
63	5h UBP1 (PF3D7_0104300) fwd	GCTGATGATTTAGTATACTTGTTTAATG
64	6a UBP1 (PF3D7_0104300) fwd	GACGATGATGATAGTATCAACGTCTCATC
65	6b UBP1 (PF3D7_0104300) fwd	GGATAAATAATAAAAAAGATGATTTAC

66	6c UBP1 (PF3D7_0104300) fwd	GTAAATAGAGACATGAGAGAAATCATG
67	6d UBP1 (PF3D7_0104300) fwd	GCATGTCGAATATATCTTTGGCTGgtgag
68	6e UBP1 (PF3D7_0104300) fwd	GAAGAAAGATTAAAAAGGAGAGGAGGTG
69	6f UBP1 (PF3D7_0104300) fwd	CCTCTAATCAAATGCTTCCGGGTGTATC
70	6g UBP1 (PF3D7_0104300) fwd	GTTTTCAAAGTTTGAATTGTAATAGAACG
71	7a UBP1 (PF3D7_0104300) fwd	CATCAGAAAATTTTAATGTAAAAG
72	7b UBP1 (PF3D7_0104300) fwd	GGATTCCAAAAAAGTATTATCTCAAAAAAA
73	7c UBP1 (PF3D7_0104300) fwd	CGCAAAATAGAATATCCGATCAAATGG
74	7d UBP1 (PF3D7_0104300) fwd	GAATCATGCAAACCGTGTGAGTCCC
75	7e UBP1 (PF3D7_0104300) fwd	GTTAACTACAGATAAGTCATATATATC
76	7f UBP1 (PF3D7_0104300) fwd	GTGGAATGAAATTATATCCCCTCCTTGTC
77	7g UBP1 (PF3D7_0104300) fwd	GGAGCAATAATACATGGTGGGATATCAGC
78	8a MyosinC (PF3D7_1329100) fwd	CGGATGAATTTTATCTTCGAAATTTAG
79	8b MyosinC (PF3D7_1329100) fwd	GCATGAGGCATCCATATTACATAGTTTG
80	8c MyosinC (PF3D7_1329100) fwd	GAAGCATTTGGTAATGCTCGAACATTAAG
81	8d MyosinC (PF3D7_1329100) fwd	CCTAGTACATCAAAATTTAGAAATCTGG
82	8e MyosinC (PF3D7_1329100) fwd	CCATAGTGGCGAATAACGAACATTACAAAAAACC
83	8f MyosinC (PF3D7_1329100) fwd	CCTTATGGTGTATTTTGTATGTTAGATG
84	8g MyosinC (PF3D7_1329100) fwd	CCTTATGGTGTATTTTGTATGTTAGATG
85	9a MyosinC (PF3D7_1329100) fwd	CGAAGTTTAAAAGCCATTGTTATTCAG
86	9b MyosinC (PF3D7_1329100) fwd	GGAAGAGTATATTAGCTAGAAAACAATTG
87	9c MyosinC (PF3D7_1329100) fwd	GGATAGTATGGAAATTATGAGTATCCAAGG
88	9d MyosinC (PF3D7_1329100) fwd	GGGACAGATCAAAGATACAAATAATAATG
89	9e MyosinC (PF3D7_1329100) fwd	GTACGTTTAGGTGATGAAGATAATTTAGG
90	10a MyosinC (PF3D7_1329100) fw	CGAAACGAGGAGACATAGGCAAAAATAAAT
91	10b MyosinC (PF3D7_1329100) fw	GTGTTTTAGGACTAGTTTGGAGTTAAAAAATG
92	10c MyosinC (PF3D7_1329100) fw	GGGAAGTGTCAACAGGTTTTTTATTAAAAG
93	10d MyosinC (PF3D7_1329100) fw	CATCACCAGCAATCATTGTAAATTCATGTG
94	11a KBI9 (PF3D7_0813000) fwd	CGAAATCAAAAAATCGACGCTCATCAACTAG
95	11b KBI9 (PF3D7_0813000) fwd	CGATAATAATTTTAATGAATTCGATGGGAATC
96	11c KBI9 (PF3D7_0813000) fwd	GCTATGGATAATGCAAATTATTATAACACC
97	11d KBI9 (PF3D7_0813000) fwd	GTGGTTCAGGATTATTACACTCAGGCAATA
98	12a Unknown (PF3D7_0609700) fw	CCCCTTGGATTAGTTTCGTATTATCGTTGTACGG

99	12b Unknown (PF3D7_0609700) fw	CCGCGTGTGGTATTACCAGTTTGAAAGGG
100	12c Unknown (PF3D7_0609700) fw	GAAAATGAGGATGATGAAGAATATGATAG
101	12d Unknown (PF3D7_0609700) fw	CGATAATTATAAGCAGCTACCGTTATG
102	12e Unknown (PF3D7_0609700) fw	GTGATGAGAGTTATCACCACAGTGATAATC
103	12f Unknown (PF3D7_0609700) fw	GAAATTGAAGAAATGAAGAAAAGGTTTAGC
104	12g Unknown (PF3D7_0609700) fw	CATCAAATTCATGTGATGATGATTTTTTTGC
105	13a Unknown (PF3D7_0609700) fw	GAGTACGAAAGAAAAAATGGACATTGTGG
106	13b Unknown (PF3D7_0609700) fw	CAGAGGATATTATAGGTGATAACCATGTG
107	13c Unknown (PF3D7_0609700) fw	GTATAAAAATGTGCGAGATGATGAGGAAAA
108	13d Unknown (PF3D7_0609700) fw	CATTTGTCCTTTAACCCCTTATGAAATATA
109	14a Unknown (PF3D7_0609700) fw	GAGTCATAAATTTGCTCAAATAAATAATTATC
110	14b Unknown (PF3D7_0609700) fw	GATTGTGATGATAATCAAATTGATTGTGG
111	14c Unknown (PF3D7_0609700) fw	CATTATCGATGAAAATTTAAAACGAATTC
112	14d Unknown (PF3D7_0609700) fw	CCACTGTGAGTTGTGTGATGCAACTTATTC
113	1n Kelch13 fw	TTATTTATTCATTCATTTATTATGTTTTTTG
114	1n Kelch13 rv	TATAAAAATAAGAACATTTAAAATTTCTTC
115	2n Formin2 fw	GATATATACTAAATATTTCTACACATATAAT
116	2n Formin2 rv	CCTTTTTACCTTTCATAAAAGGTGTCTTAC
117	3n Formin2 fw	GGAATATGATTTTTTTGAATTTAACAAAGATG
118	3n Formin2 rv	GGGAAAAAATAATAAATATAAATAAATATAAATGATTAAC
119	4n UBP1 fw	GAAGAAAATAATAATAATTAATAAAAGGAAAA
120	4n UBP1 rv	GTTGTTATTATTACTGTTCATATTATTATC
121	5n UBP1 fw	GATAATAATATGAACAGTAATAATAACAAC
122	5n UBP1 rv	CTGCCATGACACTTTGATCAGCATACATATTGC
123	6n UBP1 fw	CAGCAATATGTATGCTGATCAAAGTGTCATG
124	6n UBP1 rv	CTAAACATATCATCATATGAAATATAATG
125	7n UBP1 fw	GATGAATTTATTGGATCTTTTAAAAATAATTC
126	8n MyosinC rv	CTTTGAGGAAAGCTTTCCAAAAAATTTATTTTCC
127	9n MyosinC fw	GAAATACAAGAATATATAAATAATCTAAAG
128	9n MyosinC rv	GTAATTACAATTATTATGAGTATTATCCAAG
129	10n MyosinC fw	CTTTACATTCTAATTATTTCTTGGATAATAC
130	10n MyosinC rv	CACATATATATATATATATATATGAACAAAATTTACAATATC
131	UBP1(wt)-3xHA-T2A-Neo fwd	gctatttaggtgacactatagaatactcaagctgcggccgcTAACCTTTTTTAAAAATATGTTAACTACAG
132	UBP1(wt)-3xHA-T2A-Neo rev	GCGTAATCTGGAACATCGTATGGGTACATGGTGGTACCAAAGTACAAATCTGGAGATATGGGTGC
133	UBP1(R3138H)-3xHA-T2A-Neo fw	GGCTCCAATTAGCCCTGACCTTTATTTCcctaggACCATGTACCCATACGATGTTCCAGATTACG
136	UBP1(mutant)-3xHA-T2A-Neo rev	GTTAATAAACTTCCTCTTCCTTCTCCGTCGACAGCATAATCTGGAACATCATATGGATACATAG
137	Kelch13 codon ad fw	CTGCTGCTGGTGCTGGAGGTGCAGGTAGACCTAGGATGGAGGGTGAGAAGGTTAAGACTAAAG
138	Kelch13 codon ad rv	CTGCCATATCCCTCGAGTCATAATAACTTCGTATAATGTATGCTATACGAAGTTATAGGCCTTCAAATGTTAGCAATCAATACTG
141	K13(V520A) mutant fw	CGACAGGTTGAGGGACGTTTGGTACGCTAGTTCAAACTTGAACATTCCAAGGAGGAACAACTGCGG

142	K13(V520A) mutant rv	CCTTGGAATGTTCAAGTTTGAACTAGCGTACCAAACGTCCCTCAACCTGTCGTATACCTC
147	UBP1(mutant) Neo rev	GAAAAACGAACATTAAGCTGCCATATCCCTCGAGTTAGAAGAACTCGTCAAGAAGGCGATAG
148	1a Kelch13 rev	GTTCGAATCTAATACACTCATATCAATGG
149	11n KBI9 fw	GAAAGAAGAAATTGTACATAATAAATAAGTAAAA
150	11n KBI9 rv	GATATTACAAATGTATATATAATTTTACTGG
152	UBP1(mut)-3xHA KpnI fw	GCGAGCGTCAGAACAAGAAGAAGTCAAGCTGGTACCAGATGAACGACAGTGTTGTTACTAAGG
153	UBP1(mut)-3xHA SalI rv 1	CGGGTACATCGTAGCGTAATCTGGAACATCGTATGGGTACATGGTCCTAGGGAAATAAAGGTCAGGGCTAATTGGAG
154	UBP1(mut)-3xHA SalI rv 2	CATGTTAATAAACTTCCTCTTCCTTCTCCGTCGACAGCATAATCTGGAACATCATATGGATACATAGTCGCGTAGTCCGGCACGTCGTACGGGTACATCGTAGCGTAATCTGGA
155	12n Unknown (PF3D7_0609700) rv	CATCGTTATTTTTGTTTCCCTCACCACC
156	13n Unknown (PF3D7_0609700) fw	CCACTTTTCTGATGAAAAGGATGAATC
157	13n Unknown (PF3D7_0609700) rv	GTGATGAATTCTCAATATATCTATAATG
158	14n Unknown (PF3D7_0609700) fw	CTTATTAATGATTAATGAATATAAAC
159	2e Formin2 rev	CAACTTACGAATGGAAATCATTTC
160	2f Formin2 rev	CATTTGTTGAATTTAAATCCTTATC
161	3g Formin2 rev	GTATATTTAGGCGTTTGTTTG
162	4h UBP1 rev	CATATGAGATGTACTGTCG
163	4i UBP1 rev	CTCCTTCATAATTATCTGGAAGAG
164	4j UBP1 rev	GTATTCAAATGATTCAAGATCATC
165	6h UBP1 rev	CCTTTTAAACTTGATGAGACGTTG
166	6i UBP1 rev	GGTATAAAATATACTAATATAATATG
167	6j UBP1 rev	CTATTGGATATATTACTATCATCG
168	8h MyosinC rev	GCTATTAATAAAAATCCACATAGACC
169	8i MyosinC rev	CCTAAATAAGCACTATTTGAAGTAGC
170	9f MyosinC rev	GCTTTTAAACTTCGAAGATCACTTC
171	10h MyosinC rev	CCTTTAACATATATATCTTTATC
172	10i MyosinC fwd	CCAAATTATCAGGTCATAAAAAGG
173	10k MyosinC rev	GTGTATACATTTGAATATATTCC
174	11e UB/9 rev	GTTTACTTCTATATGAACATTTG
175	11f UB/9 rev	CATTGGATTAAAATCATCTG
176	11g UB/9 rev	GGTGAATAATTATTATTTAAGG
177	KBI5 HR fwd	GCTATTTAGGTGACACTATAGAATACTCGCGGCCGCTAAGCTATATGTAAAGGCAAATATAAAAATGC
178	KBI5 HR rev	GTTCCTCTCTATTGAAGTAACGTTTCCGAAATAAGTTGCAAATTTTTTATTTTTTCCTTC
179	KBI5 codon adj (mut) fwd	GGAAAAAATAAAAAATTTGCAACTTATTTCGGAAACGTTACTTCAATAGAGAGGAAC
180	KBI5 codon adj (mut) rev	GCACCAGCAGCAGCACCTCTAGCACGCGTTGCTTCGTGCTTTTTATGCATTAAAACTATAAGG
181	KBI6 HR fwd	GCTATTTAGGTGACACTATAGAATACTCGCGGCCGCTAAAAGAACGTTAAACAAAATATTTCAAATTTTAGAG
182	KBI6 HR rev	CTTAATACTACGGTTAATCTCTGTGCTTACCTAAAAAGAACAAAAGGAGCGGAAAATTGACAGC
183	KBI6 codon adj (mut)1 fwd	GCTGTCAATTTTCCGCTCCTTTTGTTCTTTTTAGGTAAGCACAGAGATTAACCGTAGTATTAAG
184	KBI6 codon adj (mut)1 rev	GGTGGTAAAGTGACTCCTTATCAAGAAGAC
185	KBI6 codon adj (mut)2 fwd	GAACAGTCAGATAGCAAACAGTCTTCTTGATAAGGAGTCACTTTACCACC
186	KBI6 codon adj (mut)2 rev	CCTCCAGCACCAGCAGCAGCACCTCTAGCACGCGTAAGAATCTGTTTGAAAAGGTTTATTGCG
187	KBI9 HR fwd	GGTGACACTATAGAATACTCGCGGCCGCTAATCGTCAGCAACACAATTGGGTATATCAG
188	KBI9 HR rev	GCTTGAGTCAGTAACAACCTCAACGTGCTTTCTAGTTGATGAGCGTCGATTTTTTGATTTCG
189	KBI9 codon adj (mut) fwd	CGAAATCAAAAAATCGACGCTCATCAACTAGAAAGCACGTTGAGGTTGTTACTGACTCAAGC
190	KBI9 codon adj (mut) rev	GCACCTCCAGCACCAGCAGCAGCACCTCTAGCACGCGTGAACTTCTTCTTACTGTCGAATACTGGG

191	KBI1 HR fwd	GGTGACACTATAGAATACTCGCGGCCGCTAAACTAATGTTAATAATAATATGAAC
192	KBI1 HR rev	CTTACTTACAACGTTGTTCTTAGCACTTGAAGTTATATAGGAATTATTAAAATTATCAG
193	KBI1 codon adj (mut) fwd	CTGATAATTTTAATAATTCCTATATAACTTCAAGTGCTAAGAACAACGTTGTAAGTAAG
194	KBI1 codon adj (mut) rev	GCACCAGCAGCAGCACCTCTAGCACGCGTTTCATTCATGAATATCTTAAGTTGC
195	KBI2 HR fwd	GGTGACACTATAGAATACTCGCGGCCGCTAAGTTAGCGACACAAAATCCGAGGATAG
196	KBI2 HR rev	GGTTAATCTGTGCTTTTAATAAATCAATCAACTTTAATAAATTTTGTATTTTTATTTTCATTTTGC
197	KBI2 codon adj (mut) fwd	GCAAAATGAAAATAAAAATACAAAATTTATTAAAGTTGATTGATTTATTAAAAGCACAGATTAACC
198	KBI2 codon adj (mut) rev	GCACCAGCAGCAGCACCTCTAGCACGCGTACGTACACAACTGTCTTAAGAAGGTAG
205	KBI11 HR fwd	GGTGACACTATAGAATACTCGCGGCCGCTAACAGAATAACAATGCAGTGCAATATAG
206	KBI11 HR rev	CTTATTCTGATTCTGATTCTGATTAGCTCTCATAATTTCACTCTGAAATTTTATATTATCGC
207	KBI11 codon adj (mut)1 fwd	GCGATAATATAAAATTTCAGAGTGAAATTATGAGAGCTAATCAGAATCAGAATCAGAATAAG
208	KBI11 codon adj (mut)1 rev	CCTTCTTCTCCATATTCTTCTTCTTCTCG
209	KBI11 codon adj (mut)2 fwd	GCCTATATATTTGAACGAGAAGAAGAAGAATATGGAGAAGAAGG
210	KBI11 codon adj (mut)2 rev	GCACCAGCAGCAGCACCTCTAGCACGCGTGTTTATGTTAATCTTCTCAAGTGAATATTGC
211	Formin2 HR fwd	GGTGACACTATAGAATACTCGCGGCCGCTAAGTTAATAATAATATATGGTCATCCG
212	Formin2 HR rev	GGTAGTTCTCCTTCTCACGACTACGTAACTTCCATTTTAAACTTCCTTCCATATTCG
213	Formin2 codon adj (mut) fwd	CGAATATGGAAGGAAGTTTAAAATGGAAGTTACGTAGTCGTGAGAAGGAGAACTACC
214	Formin2 codon adj (mut) rev	GCACCAGCAGCAGCACCTCTAGCACGCGTTGATGAGTTGTCTACTGATTTAAGTG
215	UBP1 HR fwd	GGTGACACTATAGAATACTCGCGGCCGCTAATTTAATGAAAAAATAGATTTTAGATATGC
216	UBP1 HR rev	CTTGTGGTTCTGAATGAATATCTTACTATATATTTTTAATTCATTTTCATCTATC
217	UBP1 codon adj S (mut) fwd	TATATATTATATTTGATAGATGAAAATGAATTAAAAATATATAGTAAGATATTCATTCAGAACCAC
218	UBP1 codon adj S (mut) rev	GGTACTTCTTAATCTTTACACCACCCATGCGAATGTCTTCTATGTTGTTTGGTGAAAC
219	UBP1 codon adj J (mut) fwd	CGCTAATAGAGTTTCACCAAACAACATAGAAGACATTCGCATGGGTGGTGTAAAGATTAAGAAGTACC
220	UBP1 codon adj J (mut) rev	CCAGCACCAGCAGCAGCACCTCTAGCACGCGTGAAATAAAGGTCAGGGCTAATTGG
221	KBI7 cod adj SEQ 1 fw	ATATACCATTTCTTATTACTGAAATTAGAC
222	KBI7 cod adj SEQ 2 fw	GAGTTCCGTAACAAAAACTACATGAGCTGC
223	KBI1 cod adj SEQ 1 fw	GCCCAAAGGAGAACATAAAGAAGAACCAC
224	KBI1 cod adj SEQ 2 fw	CTCAGGGAAAGGAGAACGCATACAAG
225	KBI5 mut SEQ fw	CAATGACAAGAGTGACCAGAATGAGAAGC
226	KBI1 cod adj SEQ 3 rev	CGTTGTCCATGTGGTTAACGTAGTCATAACC
227	KBI11 SEQ 1 fw	CGACAAGAACATACACAACAATAACAAC
228	KBI11 SEQ 2 fw	GCCTTATAGGATGCATTTCATGCATGTACG
229	KBI11 SEQ 3 fw	GCAAGACAAGTGCCACGTAGACATGGAGTG
230	Formin2 SEQ 1 fw	TTTGAATGATAAATTTGATAATCAATACATAG
231	Formin2 SEQ 2 rev	CTTAATATACTACCCTCAATACGCTCCTCG
232	Formin2 SEQ 3 fw	GAGTTGAACTACAAGTACAAAGACAAGGAC
233	KBI6 SEQ 1 fw	CGTTAACATTTTCATAACAATTAAGAGC
234	KBI6 SEQ 2 fw	CCAGTTCTCAAACTACTTCAACAACATGCC
235	KBI6 SEQ 3 fw	GAAGCACAAGCATAAAGAGATAAACGAGTAC
236	KBI6 SEQ 4 fw	CCACAGTTCAATGACTAACAAGCAGAAAAC
240	Formin2 SEQ 4 rv	CTAATGTTCCAAATCTGCATAAGTTCGC
241	5´UTR KBI2 mut intcheck fw	ATTTACATTTGTGTTGCATAAATTTGAAATC
242	KBI2 mut cod adj intcheck rv	CGTTACGAAGCTTGAAGTCAACTTCCATC
243	3´UTR KBI2 mut intcheck rv	GTCGTGTTACCCATTTTATATATTTCCTGG
244	5´UTR KBI5 mut intcheck fw	GATATATAATATTTATTTATTTTTGAAATTGTTC
245	KBI5 mut cod adj intcheck rv	GGTACTCGCACTCCCTACTAAGTATGTTCC
246	3´UTR KBI5 mut intcheck rv	ATATTCACATTCTCGTGATAAAATATTTCG

247	5´UTR KBI9 mut intcheck fw	TACATCTGTCTTTAGGAAAAAAGAAAGAAG
248	KBI9 mut cod adj intcheck rv	CTTCTCGTCGTATTCACTGTCGCTTGAG
249	3´UTR KBI9 mut intcheck rv	TTTCATCATACTCTGAATCAGAACTATCTG
253	5´UTR KBI11 mut intcheck fw	AAGAAAATAAGAAAATAAATGTAAGAAATATAAG
254	KBI11 mut cod adj intcheck rv	ATTCTGATTCTTATTCTGATTCTTATTCTG
255	3´UTR KBI11 mut intcheck rv	GGTTTTTGTTTTGGTTTTTGTTTTGGTTTTGG
256	5´UTR KBI1 mut intcheck fw	CCCCTCTTTTTTTTTTTTAATGTTCATGTG
257	KBI1 mut cod adj intcheck rv	CAGCATCGCACTTGTATGATCCACTCTTAC
258	3´UTR KBI1 mut intcheck rv	TATTTTCTGCGTCACATTTATAACTCCCCG
259	5´UTR Form2 mut intcheck fw	GGAACAATGAATAAAGCTTTTAAGGATATG
260	Form2 mut cod adj intcheck rv	CTTAATGTCAAGACTCTTGAAAATACGCTGG
261	3´UTR Form2 mut intcheck rv	CTATTCTTTCTTCATTTTTTATATCCAATG
262	KBI11 SEQ 4 fw	CCAGAAGGGTATAATATTCGACATTAACGAGG
267	15 Ap2mu (PF3D7_1218300) fw	GTTAACACGATTAGCGTCATTTGTTCTTCCG
268	15 Ap2mu (PF3D7_1218300) rv	TTTGTTGTTCCTTGTTTGAAGTAACACCCG
269	15n Ap2mu (PF3D7_1218300) fw	GTTCTTCCGTTTTTATCAATAGATTTG
270	15n Ap2mu (PF3D7_1218300) rv	CCTTGTTTGAAGTAACACCCGATTG
271	15a Ap2mu (PF3D7_1218300) fw	GAAATATGTGATGAAATAATAGATTATGG
272	15b Ap2mu (PF3D7_1218300) rv	TTTTAATATATTCACTTCTAATGTTTGTGG
273	15c Ap2mu (PF3D7_1218300) fw	TTTAATTATATAACAGGTAATTGTACATGG
274	15d Ap2mu (PF3D7_1218300) fw	GATAATGAAGAAATAATTATTGATAATTGC
275	15e Ap2mu (PF3D7_1218300) fw	AATGTTCAAGTTAAATATAAATCCATAGGC
276	MyoC(wt)-3xHA-T2A-Neo fw	gctatttaggtgacactatagaatactcaagctgcggccgcTAATCTCAGAAGATAAAATTATTTCATCATC
277	MyoC(wt)-3xHA-T2A-Neo rv	CGTAATCTGGAACATCGTATGGGTACATGGTGGTACCTACAAAAGACCTGCGCCAGAAAACTATG
278	MyoC(N277S) HR fw	gctatttaggtgacactatagaatactcaagctgcggccgcTAAGAAGGTGCAAATAAATGTGTCATAGGAAC
279	MyoC(N277S) HR rv	CGTTGTTTGACATTATACTGTTGTCAACTATACTTGCCTCTTTTACAGCTTTACAAAGTTGG
280	MyoC(N277S) cod adj fw	CATATTTTTTACCAACTTTGTAAAGCTGTAAAAGAGGCAAGTATAGTTGACAACAGTATAATGTCAAACAACG
281	MyoC(N277S) cod adj rv	GCGTAATCTGGAACATCGTATGGGTACATGGTGGTACCAACGAAGCTACGTCTCCAAAATACAATATTACCC
282	MyoC(S969P) HR fw	ttaggtgacactatagaatactcaagctgcggccgcTAATTGTTACATAGTAAGAATACATATGTATCCC
283	MyoC(S969P) HR rv	CGTTTACGTATAATGTAAGTGTATATGTAGGGACAAATCAACTTTTCAGCTTCTTGTGTCTTTTTAATTTC
284	MyoC(S969P) cod adj fw	GAAATTAAAAAGACAAGAAGCTGAAAAGTTGATTTGTCCCTACATATACACTTACATTATACGTAAACG
285	MyoC(S969P) cod adj rv	GTAATCTGGAACATCGTATGGGTACATGGTGGTACCAACGAAGCTACGTCTCCAAAATACAATATTACCC
286	MyoC(S1457L) HR fw	gctatttaggtgacactatagaatactcaagctgcggccgcTAAAATATAAAACAGAAGAAAAATAATAAAGAAC
287	MyoC(S1457L) HR rv	GAAAAGACTCTCCTCAACGTTCTTGTTACTAAGATATCCACAATCTACAATTTTTATTTGGGAAC
288	MyoC(S1457L) cod adj fw	GTTCCCAAATAAAAATTGTAGATTGTGGATATCTTAGTAACAAGAACGTTGAGGAGAGTCTTTTC
289	MyoC(S1457L) cod adj rv	CTGGAACATCGTATGGGTACATGGTGGTACCAACGAAGCTACGTCTCCAAAATACAATATTACCC
290	MyoC S969P SEQ1 fw	CCCGTTTGTGGTGATGCTAATGAAGAAGAGG
291	MyoC S969P SEQ2 fw	GGTGATTTGAAGATACGTAACACTATTCGTAACG
292	MyoC S969P SEQ3 fw	CGCAAACAAGATTACACTTCACAGCAACTAC
293	MyoC S969P SEQ4 fw	AGTCATCATCAATAATGAGTCTTAAAAACG
294	MCA2(wt)-3xHA-T2A-Neo fw	ggtgacactatagaatactcaagctgcggccgcTAATGAGTACTCACCAGATGACATCAAATTTTTATCAC
295	MCA2(wt)-3xHA-T2A-Neo rv	CTGGAACATCGTATGGGTACATGGTGGTACCGGAAACACATTTAATATTCAAATCGATAATACC
296	MyoC(D500G) HR fw	gacactatagaatactcaagctgcggccgcTAAGAATCAACAAAATATGTTATGAAATTTTTAGC

297	MyoC(D500G) HR rv	GTCAAGAACTCCGCAGAAAAGGTTAATGCCTTTTATATATCCTATAGATTCATTTGTTCTTTCAACC
298	MyoC(D500G) cod adj fw	GAAAGAACAAATGAATCTATAGGATATATAAAAGGCATTAACCTTTTCTGCGGAGTTCTTGACATATTCG
299	MyoC(Q635H) HR fw	ggtgacactatagaatactcaagctgcggccgcTAAGATCATATAGAAAATAACATTTGTGAGGAACCC
300	MyoC(Q635H) HR rv	CTCTAAGAATCCTGTGCTGTTGTAAACTACGTGCCCAGCAAAATGTACAATAATAAAACTACTAG
301	MyoC(Q635H) cod adj fw	GTAGTTTTATTATTGTACATTTTGCTGGGCACGTAGTTTACAACAGCACAGGATTCTTAGAGAAG
302	MCA2 (Y1334.)-GFP fwd	ggtgacactatagaatactcgcggccgctaaAATAATTTTAGCAAACCAAATTTTTTAGATAAATTTTTTTATG
303	MCA2 (Y1334.)-GFP rev	CAGCACCAGCAGCAGCACCTCTAGCacgcgtTTTTTTTAATTGTTCATATAACTTTTTATTTTTGGTC
304	MCA2(Y1344.)-3xHA fw	ggtgacactatagaatactcaagctgcggccgcTAAAATAATTTTAGCAAACCAAATTTTTTAGATAAATTTTTTTATG
305	MCA2(Y1344.)-3xHA rv	CTGGAACATCGTATGGGTACATGGTGGTACCTTTTTTTTAATTGTTCATATAACTTTTTATTTTTGGTC
306	UBP1 mut SEQ1 fw	CGTTCATTTATAAAATTAATATTTAATTGG
307	UBP1 mut SEQ2 fw	CATTCCTTTCTTCAAGAAGTACTACTTCC
308	UBP1 mut SEQ3 fw	CTTCAACGTTAAGGACTTCATTACTAACC
309	UBP1 mut SEQ4 fw	GGACGCAGTTAACTCAGTTAACCACGTTAAC
310	Intcheck UBP1(wt)-3xHA fw	GTCAAATGCATCAAGAGAATACGAATAGTG
311	Intcheck UBP1(wt)-3xHA rv	GCCTATACAATATTTATGTTTAATTTTTGTTCGC
312	16 Ap2a (PF3D7_0617100) fw	GTAACCATAAATTATATAATAAATAAAAGACACAAC
313	16 Ap2a (PF3D7_0617100) rv	GATCTGATAAATATCTATAACACTTCATAGC
314	17 Ap2a (PF3D7_0617100) fw	GAAAGTAACAATAGTAATAATAATAGCAAC
315	17 Ap2a (PF3D7_0617100) rv	TAAAATATTACAATAATAAAAAGAGAACAG
316	16n Ap2a (PF3D7_0617100) fw	TAAATAAAAGACACAACAACAATAATTACAC
317	16n Ap2a (PF3D7_0617100) rv	CACTTCATAGCTGCATAAGTTTGTACTTTC
318	17n Ap2a (PF3D7_0617100) fw	GTAATAATAATAGCAACAATAATAACAACAAC
319	17n Ap2a (PF3D7_0617100) rv	CAATAATAAAAAGAGAACAGATGTTATAGATTG
320	16a Ap2a (PF3D7_0617100) rv	CATTATTTTTTATATCTGCTTTTATGGTAC
321	16b Ap2a (PF3D7_0617100) fw	GAGTCAATGTATTATTATATAAAAAAGGAAG
322	16c Ap2a (PF3D7_0617100) fw	CATTGAATATGGTATTATTTATGAATGTTCC
323	17a Ap2a (PF3D7_0617100) fw	tatttttcagACATGCCGTTATTGACTTTAACG
324	17b Ap2a (PF3D7_0617100) fw	CTCACTGTATCAGACATCTTTTACAATATACCC
325	17c Ap2a (PF3D7_0617100) fw	ttggtagATTGGAATGCTCATATGCGCATC
326	18 MCA2 (PF3D7_1438400) fw	CATTGATGTTTCCTCTAGGGAGAATTTCTTCC
327	18 MCA2 (PF3D7_1438400) rv	GTATTATCACTAGGTAAGAAAACGCCACTGTTTGG
328	18n MCA2 (PF3D7_1438400) fw	GAATTTCTTCCAACATGATATTCAAAATACAAATC
329	18n MCA2 (PF3D7_1438400) rv	GATTTAAAATATTAATATTATTCAAAGTTTTCTCATTATCC
330	18a MCA2 (PF3D7_1438400) fw	CTTGATATAACACAAAATGAAAAACCAAACGC
331	18b MCA2 (PF3D7_1438400) rv	GTACGAATCTTTCATAGTGTAATAGGAACCCG
332	MyoC mut HR fwd	gctatttaggtgacactatagaatactcgcggccgctaaACAAAATCGAGTGTATATGAATTAAATGAAG
333	MyoC mut HR rev	CTTCTCTTCCTCCTCGTTTGCGTCGTCGCATACGGGATCATATACAATATCTTTCATATTATTATTATTATTGTTCG
334	MyoC mut cod adj (mut) fwd	CGAACAATAATAATAATAATATGAAAGATATTGTATATGATCCCGTATGCGACGACGCAAACGAGGAGGAAGAG

335	MyoC mut cod adj (mut) rev	GCACCAGCAGCAGCACCTCTAGCacgcgtAACGAAGCTACGTCTCCAAAATACAATATTACCC
336	MCA2 mut HR fwd	gctatttaggtgacactatagaatactcgcggccgctaaAATAGTATGAACACATATTCCCCTTTATATAGTTCC
337	MCA2 mut HR rev	GCTGGTTAATCTCGTAAGGAAGCTCGTTGTATAAGTAATTCCTAATTAACATATCAGCTGACC
338	MCA2 mut cod adj (mut) fwd	GGTCAGCTGATATGTTAATTAGGAATTACTTATACAACGAGCTTCCTTACGAGATTAACCAGC
339	MCA2 mut cod adj (mut) rev	CAGCACCAGCAGCAGCACCTCTAGCacgcgtACTTACGCACTTTATGTTAAGGTCTATTATTCC
340	MyoC S1457L SEQ1 fw	GAATAGTGGATATTCTTGTATGTGGACCAAGG
341	K13 (V589I) mut rv	GTTCAACCTTTCTCCGTTTGTACCTCCTATAATGTATATCTTGTTGTCGAATGCTACGCACATTGC
342	K13 (V589I) mut fw	CAATGTGCGTAGCATTCGACAACAAGATATACATTATAGGAGGTACAAACGGAGAAAGGTTGAAC
343	Kelch13 SEQ1 fw	CTTGAACATTCCAAGGAGGAACAACTGCGG
344	K13 (E612K) mut rv	CCTTGCCTCCAACAATGCGTAAGGGAACTGCTTCCACTTGTTCATCTTCTCCTCGTAAACCTC
345	K13 (E612K) mut fw	GAGGTTTACGAGGAGAAGATGAACAAGTGGAAGCAGTTCCCTTACGCATTGTTGGAGGCAAGG
346	5´ MyoC(S969P) intcheck fw	CTACTGGTTTTCTTGAAAAAAATAAGGATCAG
347	3´ MyoC(S969P) intcheck rv	CAGCAATTTCTTTTATGTAAAAATCTCTTTC
348	MyoC (S969P) intcheck ca rv	GCCTGAATCTTTGAAGCGTAGTAGTTCTGACGG
349	5´UTR UBP1 mut intcheck fw	GAAATCTTTGAAAAATATTTTAATGCTGATG
350	UBP1 mut cod adj intcheck rv	GAACTGGTTGAAGTTAATCTTGTGGTTCTG
351	3´UTR UBP1 mut intcheck rv	CCCAAAATAATATACACATAATATTCCATATAG
360	MyoC mut SEQ 1 fwd	GTAAAATTATAAGTAAGCATACATCGAGTAG
361	MyoC mut SEQ 2 fwd	GAACATTAAGCAAAAAAAGAACAACAAGGAG
362	MyoC mut SEQ 3 fwd	CTTCGTAAGAACAAAAAGTTAGTTAACAACG
363	MyoC mut SEQ 4 fwd	GGAATACAGAACTCAAACAAAAAAAACCAGC
364	MyoC mut SEQ 5 fwd	GTCTTCTTAGGATTGTTAACCTTAACTCAGG
365	5´UTR MyoC mut intcheck fw	GAAAATAGATTTTACAGATTTTAAAGAACATGTAC
366	MyoC mut cod adj intcheck rv	GCTCCAAATAAAAAGGTTATTCTTCTCTTCC
367	3´UTR MyoC mut intcheck rv	GAATAAATTGTTTTTTTCCTCTTCTTCATTAGC
368	AP2a-16d-fw	GTATTTAGATATAATAAAATTGATAAGTAG
369	AP2a-16e-rev	CATTGACTCATATTTTTGAAATAATTA
370	AP2a-16f-rev	CTACTTTTATTGTTATTATTATAATC
371	AP2a-16g-rev	CAATTTCTTCAATATTAAATGTACACTC
372	AP2a-17d-rev	GTATATGCATTTATGTGTTTGC
373	AP2a-17e-rev	CATATATATATATATATATTATCC
374	AP2a-17f-rev	CTATATTATTCTTATTTATGAACATTTATG
375	AP2a-17f-fw	CATAAATGTTCATAAATAAGAATAATATAG
376	AP2a-17g-rev	CAATACACTTTAAATATTTTAAAACTTG
377	AP2a-17h-fw	GATCTATATAAAATAATACCATATTATAC
382	Eps15 HR mut C-term fw	gtgacactatagaatactcgcggccgctaagACGATGTAACTTTTCGACAGAATGCTGATG
383	Eps15 HR mut C-term rv	CGTTGTCGTTGTTGTTGTCGTAAGTGTTGTTATTATTATTATTATCATATGTATTATTATTATTATTATTATCATATG
384	Eps15 CA (mut C-term) fw	GATAATAATAATAATAATAATACATATGATAATAATAATAATAACAACACTTACGACAACAACAACGACAACG
385	Eps15 CA (mut C-term) rv	GCACCAGCAGCAGCACCTCTAGCacgcgtTGATGAGTTGTCTACTGATTTAAGTGAGTTG
386	Eps15 mut SEQ5 fw	GAGATAAAAATAAAAAGAAGATGCACGGTAAGGG
387	Eps15 mut SEQ6 fw	CATGATTGACTACCTTAAGAAGCAGAAGCAGCG
388	5´UTR Eps15 mut intcheck fw	catttttatttcaatttattattccttcc
389	Eps15 mut CA intcheck rv	GTCAACCATTCCGTTGTCGTTGTTGTTGTCG
390	3´UTR Eps15 mut intcheck rv	CAAATGGAAATCTTTATCCACCATACCATTATC
391	K13(V520I) mutant rv	CCTCCTTGGAATGTTCAAGTTTGAACTTATGTACCAAACGTCCCTCAACCTGTCGTATACC
392	K13(V520I) mutant fw	GGTATACGACAGGTTGAGGGACGTTTGGTACATAAGTTCAAACTTGAACATTCCAAGGAGGAAC
395	Myo N277S SEQ1 fw	GCTTTACATGGAGGAGGGAATAAGGTGGGACCC

396	Myo N277S SEQ2 fw	GTAAAAAGAATAAGAAGATGCAGGACACTACAAC
397	Myo N277S SEQ3 fw	GAAATCAATTCTTGCACGTAAGCAGCTTCGTCG
398	Myo N277S SEQ4 fw	TATTAGAGAAGGTTCGGGTGTGTGATCAAC
399	Myo (S1457L) BlpI fw	ACAGGACAACATTAACAAAAACTACACTTTGCTTAGCGTTGGTTGCAAAGACGGAGTAATTTAC
400	Myo D500G SEQ1 fw	TGGATGTTTATTTTTAAAAGTGGTTGAAAG
401	MCA2 mut SEQ1 fw	ccttatatttatatatgttgtgcatttgttgaac
402	MCA2 mut SEQ2 fw	GTGAATAATATGAATAATATGAATAATGTG
403	MCA2 mut SEQ3 fw	GAAAAACAGTAATCGTGCAGTAGTTATGCC
404	MCA2 mut SEQ4 fw	GTAAATAACGTAGACAAGAAGAACAACGACG
405	MCA2 mut SEQ5 fw	GAGAACAGTTTGTACAACTTCACTACTCACC
406	MCA2 mut SEQ6 fw	CTATACAATGAAGGACAGTTATATTAGTAGTC
407	MCA2 mut SEQ7 fw	AAGAAGCTTCAGGGTGGTAAGCAGCTTTGC
408	Intcheck 5UTR MCA2 wt fw	GGAATATAATTTGAAATATGTAAAATTTAAACTGTCC
409	Intcheck 3UTR MCA2 wt rv	CATAAACACAAAAATTTAAAGATGGAGTGG
410	Intcheck 5UTR MyoF wt fw	CAAATTATCAGGTCATAAAAAGGCAATAACATG
411	Intcheck 3UTR MyoF wt rv	ATATATATATATATGAACAAAATTTACAATATC
412	Intcheck 5UTR MyoF 277 fw	AAAAATTTTATTTAATATATAAACAAATTGTAGGG
413	Intcheck 3UTR MyoF 277 rv	CATTTTCATTATTACTCATAATTGAATTATCC
414	Intcheck MyoF 277 CA rv	GTTCTCAACATCGTCGTTCTCGTTGTTTGAC
416	Intcheck 5UTR MCA2 (Y1344.) fw	ATCCATAACAATAATAATAATATTGAGTGG
417	Intcheck 3UTR MCA2 (Y1344.) rv	AACTTTTTTGTTTTGGTATCTTTGTCAAGC
418	Intcheck 5UTR MyoF (S1457L) fw	CGAATGAGTGTATTTCACCGAGGCATGTATC
419	Intcheck 3UTR MyoF (S1457L) rv	GTAGCATCAAATACTACAATTATACATATTG
420	Intcheck MyoF 1457 CA rv	GTTCTTAATGTAGTTGAAAAGACTCTCCTC
421	Intcheck 5UTR MyoF (Q635H) fw	CGAAAATTATGTAAATTATAAATGTGATGAAG
422	Intcheck 3UTR MyoF (Q635H) rv	TTTTTTCAAGAAAACCAGTAGAATTATACAC
423	Intcheck MyoF 635 CA rv	CTTTGTTCTTCTCTAAGAATCCTGTGCTGTTG
424	Intcheck 5UTR MyoF (D500G) fw	CAAACAATATTAATTAGTGGTGAATCAGGTGC
425	Intcheck 3UTR MyoF (D500G) rv	GATTCAAATCCAAAGATATCTAATACACCAC
426	Intcheck MyoF 500 CA rv	CTCGAAACCGAATATGTCAAGAACTCCGCAG
428	HR MyoF wt (N-term) fw	ggtgacactatagaatactcgcggccgcTAAGAAGGTGCAAATAAATGTGTCATAGGAACAAAG
429	HR MyoF wt (N-term) rv	CCTCTTCTGATATTAACTTCTGCTCGTTTAAACCTGAAGGAGCAGACAAACCACTCGAATCAAATAAG
432	1g Kelch13 (PF3D7_1343700) rv	CAAATAAAGCCTTATAATCATAGTTATTACCACC
433	5UTR KBI6 mut intcheck fw	GATTTGATTATAAAATAGATCTTATAACCAGG
434	KBI6 mut cod adj intcheck rv	CTCGAATAACTTAATACTACGGTTAATCTC
435	3UTR KBI6 mut intcheck rv	CTTACGGTACCATCTTTTTTGTGTTCTCTCTC
437	Intcheck 3` MyoF 277 rv neu	CATCACATTTATAATTTACATAATTTTCGACGTC
439	5UTR KBI11 mut intcheck fw neu	TTTTTTTTTTTGATGTACAATAATTAAAGAG
440	3UTR KBI11 mut intcheck rv neu	GATGACTATTATTAATATTGTTGTTGATGTTG
441	5 intcheck MyoF (Q635H) fw neu	CAATTATGAGTAATAATGAAAATGATGACGTCG
442	3UTR intcheck MyoF (Q635H) rv neu	CAATTTCTGTGCATCTAAAGATAACTGATCC

443	MCA2 mut SEQ8 rv	GGTTTGAGTTATAGTACTGCATTATGTTACGG
444	MCA2 mut HR NEU fwd	gctatttaggtgacactatagaatactcgcggccgctaaAGTGAAAATGATAGAAACGAATCTATACAG
445	MCA2 mut CA SnaBI rev	GCAGCCCTAGTAATGTTTGAAGTTTTTACTACGTAGTCATTCATATTACGGTTGTTGTTTGAATATATCTTC

Appendix D

Appendix D.1 Mutations found in the different candidates by sequencing of field isolate DNA obtained from the Fever without source study in Ghana (PCR and sequencing was kindly performed by Birgit Förster) [*Hogan et al. 2018*]. Prevalence (%) is indicated in case, they were already listed in MalariaGEN (*Pf* Community Project).

Candidate	Mutation	Type	Base exchange	West Africa	Central Africa	East Africa	South Asia	SEA (West)	SEA (East)
Kelch13 (PF3D7_1343700)	no mutation found								
UBP1 (PF3D7_0104300)	N1710S	non-syn	AAC/AGC						
	K1914N	non-syn	AAA/AAC						
	E1915K	non-syn	GAA/AAA						
	1283F	syn	TTT/TTC	99,8	99,7	100	100	100	100
MyosinF (PF3D7_1329100)	N277S	non-syn	AAC/AGC	32,9	38,6	41,5	71,4	67	54,8
	I568V	non-syn	ATA/GTA	79,7	70,9	72,4	90,4	99,7	100
	C1196S	non-syn	TGC/AGC	99,9	100	100	100	100	100
	H1587R	non-syn	CAT/CGT						
	N1615K	non-syn	AAC/AAA						
	M1872L	non-syn	ATG/CTG	93,2	91,6	94,7	84,8	99	94,3
	T1930S	non-syn	ACA/TCA	94,5	93,8	95,9	86,7	99	94,9
	1896S	syn	TCA/TCT	93,2	92,2	95,5	87	99	94,9
	1728S	syn	TCC/TCT	87,8	89,8	89,9	98,6	100	100
	1870S	syn	AGC/AGT	93,2	91,6	94,7	84,5	99	94,3
KIC6 (PF3D7_0609700)	Y1215C	non-syn	TAT/TGT	34,6	27,7	31,4	51,6	82,9	96,8
	D1651N	non-syn	GAT/AAT						
	K1652Q	non-syn	AAA/CAA						
	D1658N	non-syn	GAT/AAT						
	K1659Q	non-syn	AAA/CAA						
	D1672N	non-syn	GAT/AAT						
	D1675N	non-syn	GAT/AAT						
	1848N	syn	AAT/AAC	39,7	36,3	38,6	11,7	1,5	3,5
	1671D	syn	GAT/GAC						
KIC7 (PF3D7_0813000)	no mutations found								

Candidate	Mutation	syn/non-syn	Codon						
Ap2μ (PF3D7_1218300)	no mutations found								
Ap2a (PF3D7_0617100)	709N	syn	AAT/AAC						
MCA2 (PF3D7_1438400)	1435E	syn	GAG/GAA	21	14,9	14,8	50,4	75,3	71,2
Eps15 (PF3D7_1025000)	F486S	non-syn	TTT/TCT	90,7	92,5	93,3	97,5	99,1	98,9

Appendix D.2 Prepared multipools that were not finished until the end of the thesis. Recodonized sequence was ordered and produced by GenScript®.

Candidate	Mutation	Reference
*Pf*KIC6 (PF3D7_0609700)	C1689F	MalariaGen
	D232Y	MalariaGen
	E605Q	MalariaGen
	F181S	MalariaGen
	G1078D	MalariaGen
	G358C	MalariaGen
	H1480R	MalariaGen
	H479Q	MalariaGen
	H498Q	MalariaGen
	K1686Q	MalariaGen
	K571I	MalariaGen
	L501F	MalariaGen
	L819I	MalariaGen
	N1141D	MalariaGen
	N1685D	MalariaGen
	N1688D	MalariaGen
	N712S	MalariaGen
	Q1516K	MalariaGen
	R550K	MalariaGen
	Y1215C	MalariaGen & Sequencing Ghana
	Y506C	MalariaGen
*Pf*Eps15 (PF3D7_1025000)	S152R	MalariaGen
	E354D	MalariaGen
	D355N	MalariaGen
	N365I	MalariaGen
	H436Y	MalariaGen
	Y444D	MalariaGen
	G452C	MalariaGen
	F486S	MalariaGen & Sequencing Ghana
	K507T	MalariaGen
	I650K	MalariaGen
	M846I	MalariaGen
	T849M	MalariaGen
	R859K	MalariaGen

		A898S	MalariaGen
		E929Q	MalariaGen
		Q932E	MalariaGen
*Pf*MCA2 (PF3D7_1438400)		D226E	MalariaGen
		T254I	MalariaGen
		V723G	MalariaGen
		P748R	MalariaGen
		D777V	MalariaGen
		V919G	MalariaGen
		L1132V	MalariaGen
		E1339A	MalariaGen
		V1764A	MalariaGen

Appendix D.3 Schemes of multipools corresponding to Appendix D.2.
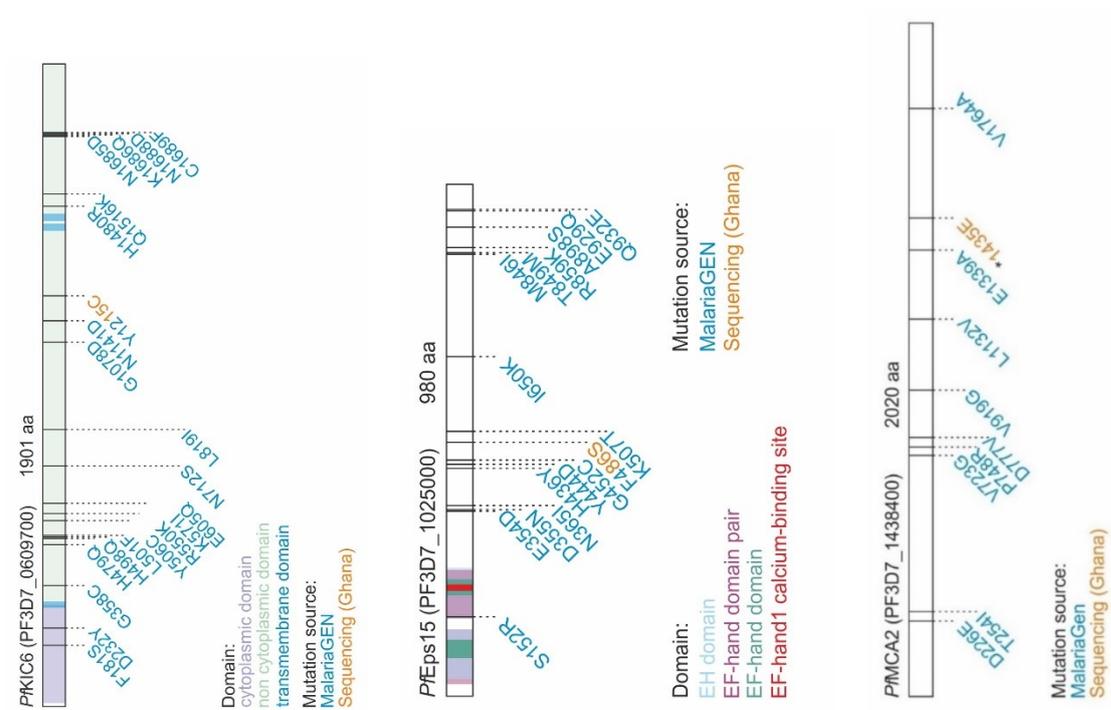
Appendix E

Appendix E.1 Gating strategy growth curve (FACSDiva Version 6.1.3).
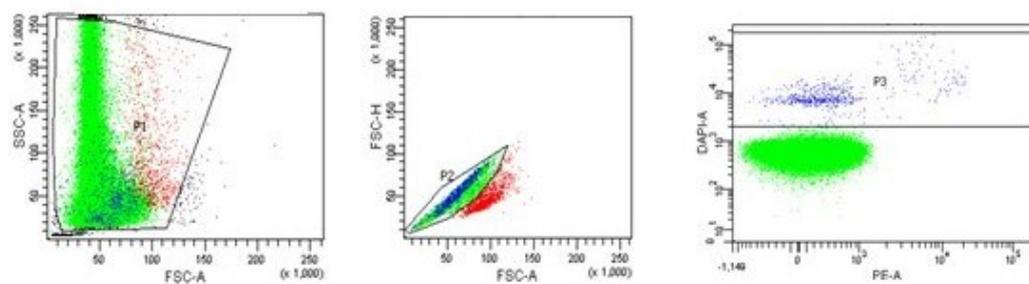
Appendix E.2 Gating strategy fitness assay (FACSDiva Version 6.1.3).
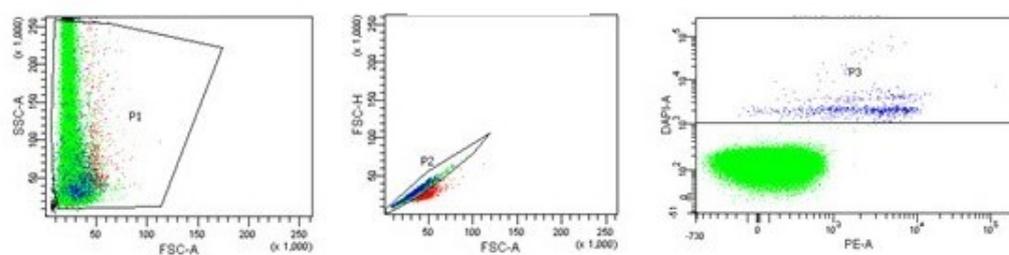
Appendix F

Appendix F.1 Integration checks of cell lines transfected with SLI-plasmids.
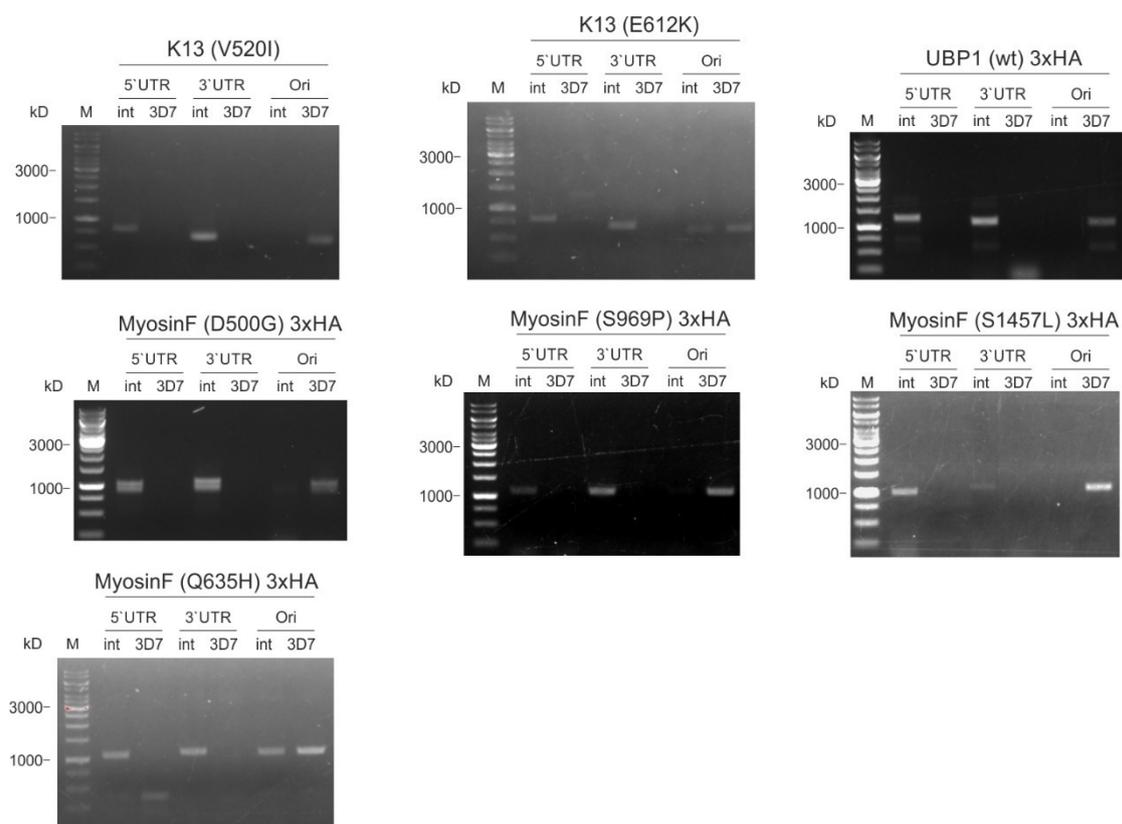
Appendix F.2 Western blot analysis of different mutants obtained via SLI.

Appendix G

Appendix G.1 Confocal microscopy images of K13C580Y parasites after DHA removal in RSA. Three different time points were imaged, and development of cells was followed-up. Corresponding calculations are listed in Appendix G.2.

+ DHA
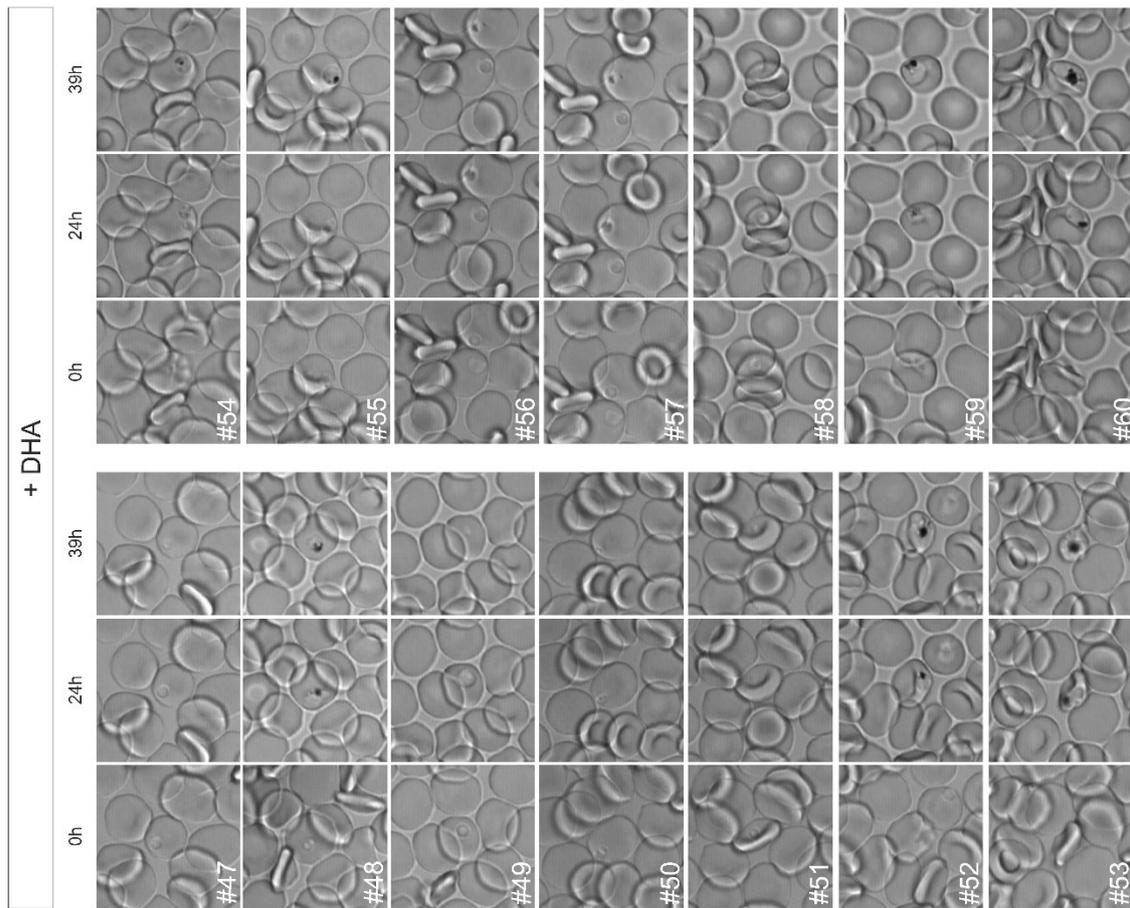
Appendix G.2 Assessment of development of K13^{C580Y} parasites after DHA removal. Three different timepoints were imaged by confocal microscopy (see Appendix G.1).

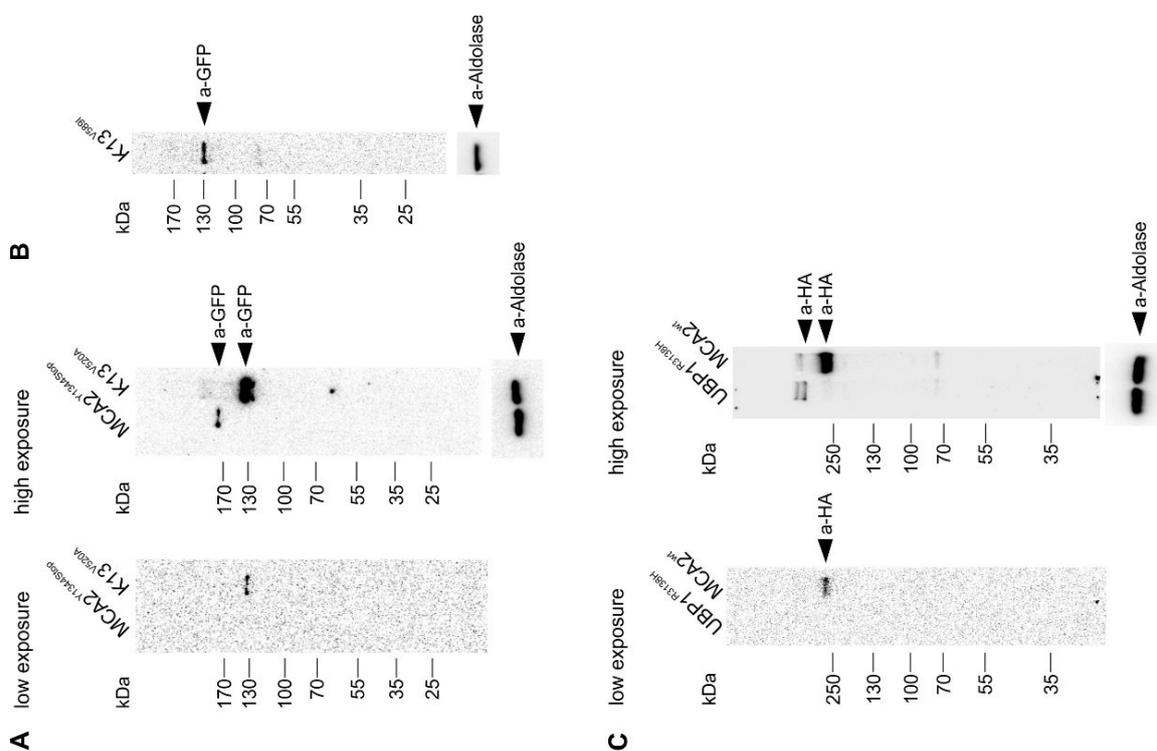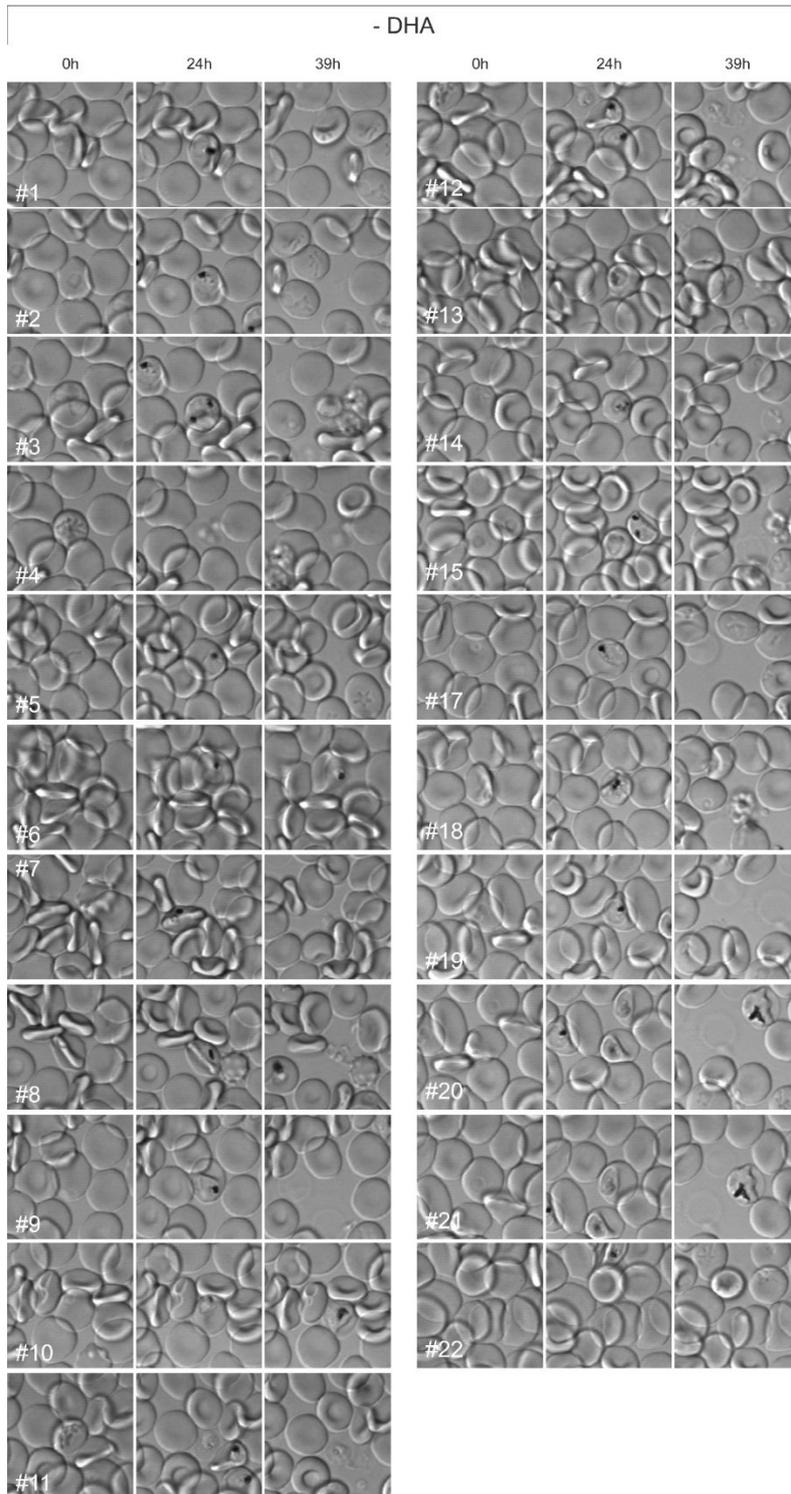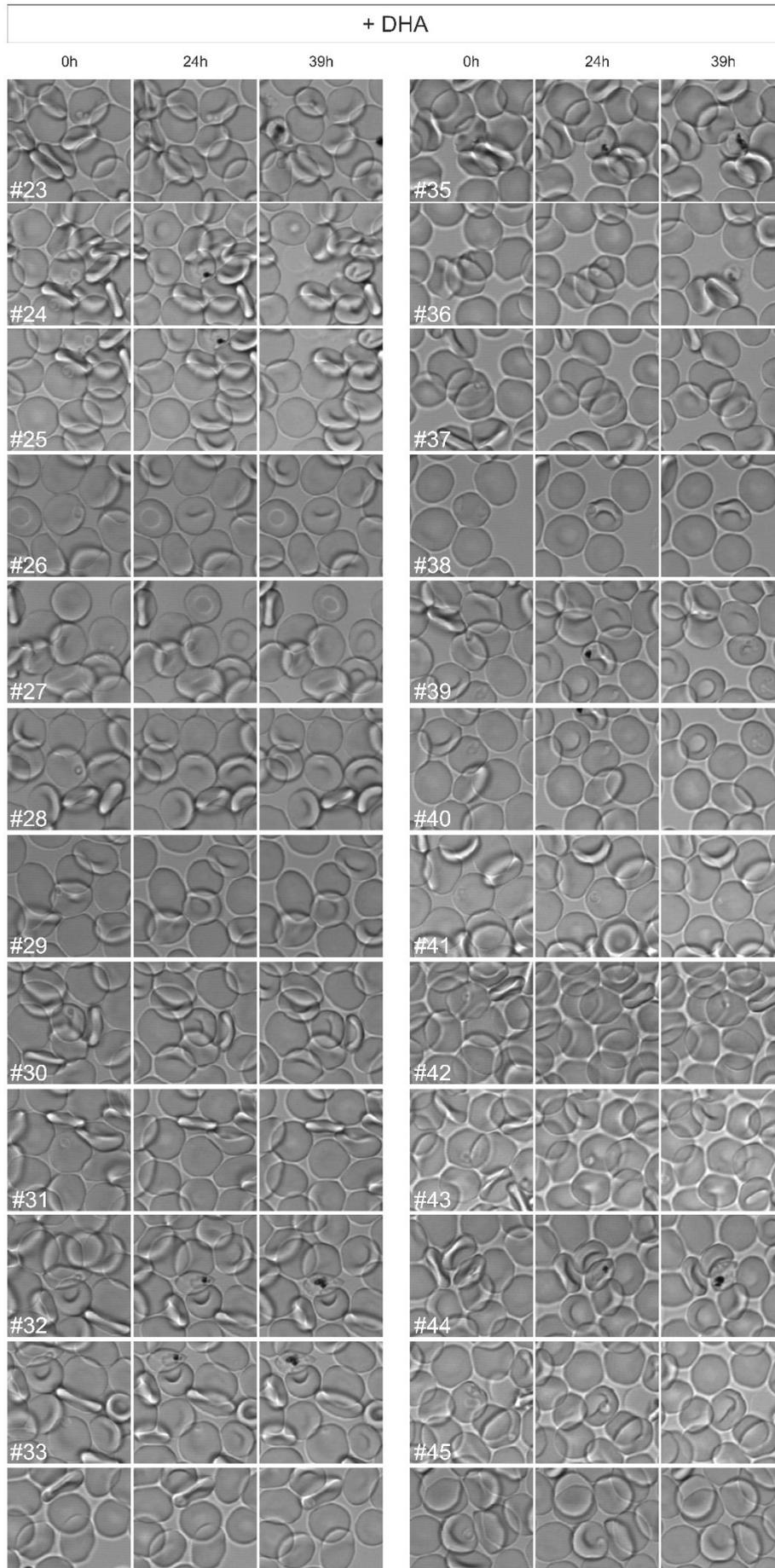|	Treatment	Parasite (K13^{C580Y})	39 h after DHA removal				24 h after DHA removal			follow-up of parasites (measured area at 24h)	remarks
			reinvasion	delayed growth	dead		area [µm^2]	Min	Max		
	control	#1	1	0	0		23,875	25	164	reinvasion	
		#2	1	0	0		25,203	29	191	reinvasion	
		#3	1	0	0		17,25	27	183	reinvasion	
		#4					1,078	92	111		cell destroyed by method after 39h
		#5	1	0	0		17,594	41	155	reinvasion	
		#6					17,094	31	149		cell destroyed by method after 39h
		#7	1	0	0		22,078	25	182	reinvasion	
		#8	1	0	0		26,031	32	200	reinvasion	
		#9					25,25	30	150		cell destroyed by method after 39h
		#10	0	1	0		10,172	53	145	late schizont	
		#11	0	0	1		8,125	75	133		cell destroyed by method after 39h
		#12	1	0	0		20,516	31	130	reinvasion	
		#13	1	0	0		14,531	57	172	reinvasion	
		#14	1	0	0		11,375	44	144	reinvasion	
		#15	1	0	0		14,5	34	216	reinvasion	
		#16					3,219	76	136		parasite dead after 24h
		#17	1	0	0		17,453	27	169	reinvasion	
		#18	1	0	0		28,703	37	193	reinvasion	
		#19	1	0	0		19,141	32	166	reinvasion	
		#20	1	0	0		15,719	62	220	reinvasion	
		#21	0	1	0		13,641	77	159	late schizont	
		#22	1	0	0		11,828	36	225	reinvasion	
Summe (39h)		**17**	**15**	**2**	**0**	**Summe (24h)**	**17**	**18**			
Reinvasion [%]			88								
Troph/schizont [%]				12							
Dead [%]					0						

	Treatment	Parasite (K13^{CS80Y})	39 h after ART removal					24 h after DHA removal			follow-up of parasites (measured area at 24h)	remarks
			reinvasion	delayed growth	dead			area [μm²]	Min	Max		
	DHA	#23	0	1	0			11,016	74	157	late schizont	
		#24						12,438	26	153		cell destroyed by method after 39h
		#25	0	0	1			0,312	139	154		parasite dead after 24h
		#26	0	0	1			0,953	89	116		parasite dead after 24h
		#27	0	0	1			4,391	75	132	dying ring	
		#28	0	0	1			0,156	100	106		parasite dead after 24h
		#29	0	0	1			0,266	116	144		parasite dead after 24h
		#30	0	0	1			0,203	104	110		parasite dead after 24h
		#31	0	0	1			0,188	96	100		parasite dead after 24h
		#32	0	1	0			9,688	28	127	late schizont	
		#33	0	0	1			0,75	93	123		parasite dead after 24h
		#34	0	1	0			3,859	83	192	arrest in ring	
		#35	0	1	0			12,688	38	142	late schizont	
		#36						4,281	78	140		cell destroyed by method after 39h
		#37	0	0	1			0,219	99	107		parasite dead after 24h
		#38	0	0	1			3,531	79	128	dying ring	
		#39	1	0	0			15,5	27	212	reinvasion	SURVIVOR
		#40	0	0	1			1,688	104	135		parasite dead after 24h
		#41	0	0	1			4,781	101	136	dying ring	
		#42	0	0	1			0,328	112	122		parasite dead after 24h
		#43	0	0	1			4,719	95	172	dying ring	
		#44	0	1	0			12,328	41	174	late schizont	
		#45	0	0	1			5,859	80	183	dying ring	
		#46	0	0	1			1,156	102	131		parasite dead after 24h
		#47	0	0	1			3,828	109	147	dying ring	
		#48	0	0	1			14,141	46	143	dying trophozoite	
		#49	0	0	1			9,562	93	158	dying ring	
		#50	0	0	1			2,312	76	118	dying ring	
		#51	0	0	1			0,719	94	107		parasite dead after 24h
		#52	0	1	0			8,422	28	144	late schizont	
		#53	0	1	0			11,734	39	147	late schizont	
		#54	0	1	0			5,219	73	115	young trophozoite	
		#55	0	1	0			7,828	66	155	young trophozoite	
		#56	0	1	0			4,484	95	135	arrest in ring	
		#57	0	0	1			0,391	80	133		parasite dead after 24h
		#58	0	0	1			4,953	73	152	dying ring	
		#59	0	1	0			8,125	52	130	young trophozoite	
		#60	0	1	0			13,109	34	142	late schizont	
Summe (39h)		36	1	12	23	Summe (24h)	36	8				
Reinvasion [%]			3									
Troph/schizont [%]				33								
Dead [%]					64							
						Late schizont	7	19				
						Dying ring	9	25				
						arrest in ring	2	6				
						reinvasion	1	3				
						dying troph	1	3				
						young troph	3	8				
						dead	13	36				
						Summe	36	[%]				

Appendix H

Appendix H.1 Raw curves of three independent fitness assays performed in RPMI complete medium. Corresponding calculations are listed in Appendix H.2.
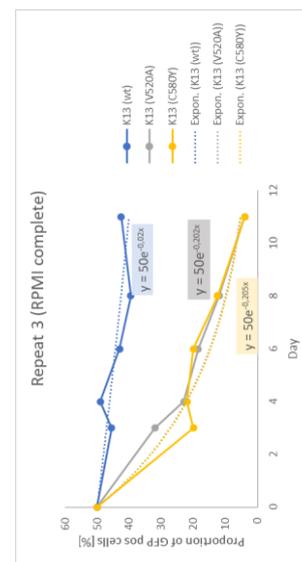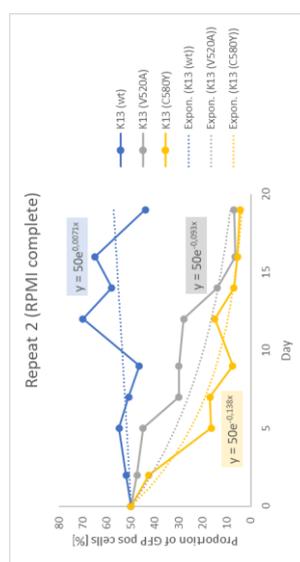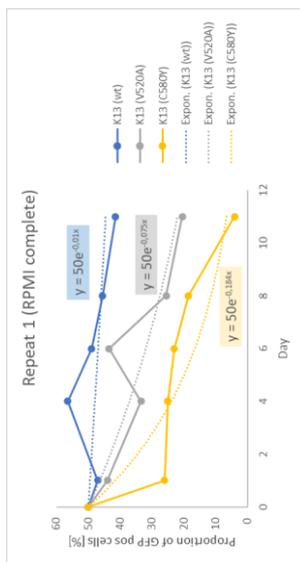
Appendix H.2 Calculation of fitness costs per generation of different mutations grown in RPMI complete medium. Corresponding raw curves are listed in Appendix H.1
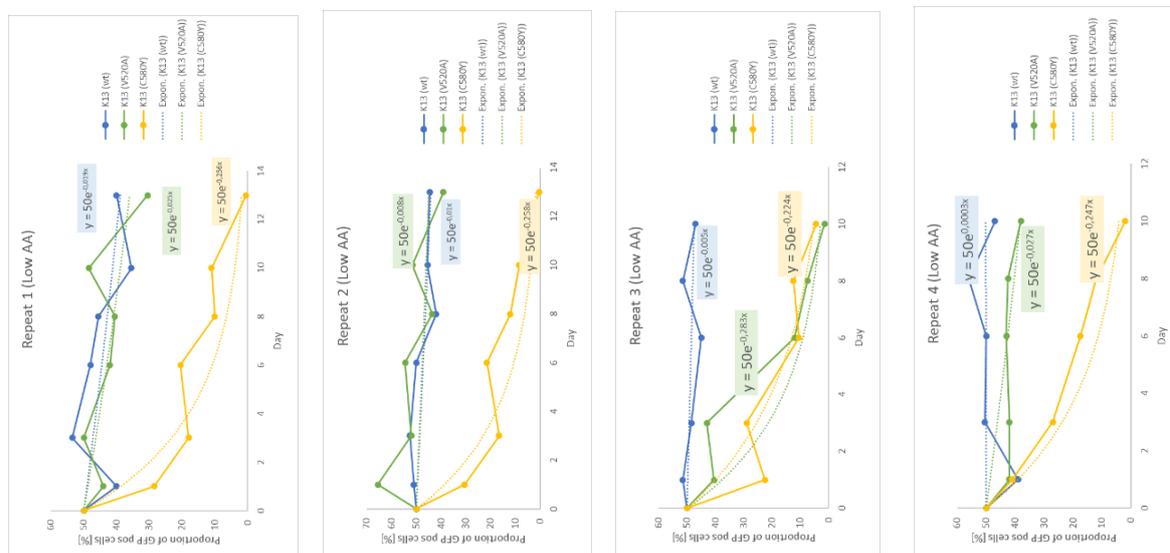
Repeat 1	Factor from trend line	retained growth per day	% loss per day	factor per generation	retained growth /generation
WT	-0,010	0,990	0,995	-0,02	0,980198673
520	-0,075	0,928	7,226	-0,15	0,860707976
580	-0,184	0,832	16,806	-0,368	0,692117182

Repeat 2	Factor from trend line	retained growth per day	% loss per day	factor per generation	retained growth /generation
WT	0,0071	1,007	-0,713	0,0142	1,014301299
520	-0,093	0,911	8,881	-0,186	0,830273595
580	-0,138	0,871	12,890	-0,276	0,758812931

Repeat 3	Factor from trend line	retained growth per day	% loss per day	factor per generation	retained growth /generation
WT	-0,02	0,980	1,980	-0,04	0,960789439
520	-0,202	0,817	18,291	-0,404	0,667644121
580	-0,205	0,815	18,535	-0,41	0,66365025

	growth factor /day					Repeat	growth factor/generation*		
Repeat	WT	V520A	C580Y				WT	V520A	C580Y
1	0,990	0,928	0,832			1	0,980	0,861	0,692
2	1,007	0,911	0,871			2	1,014	0,830	0,759
3	0,980	0,817	0,815			3	0,961	0,668	0,664
mean	**0,992**	**0,885**	**0,839**			mean	**0,985**	**0,786**	**0,705**
sd	0,011	0,049	0,024			sd	0,022	0,085	0,040

*assumption: 1 generation=2 days

							fitness cost per generation		
						Repeat	WT	V520A	C580Y
						1	0,020	0,139	0,308
						2	-0,014	0,170	0,241
						3	0,039	0,332	0,336
						mean	**0,015**	**0,214**	**0,295**
						sd	0,022	0,085	0,040

Appendix H.3 Raw curves of three independent fitness assays performed in low AA medium. Corresponding calculations are listed in Appendix H.4.

Appendix H.4 Calculation of fitness costs per generation of different mutations grown in low AA medium. Corresponding raw curves are listed in Appendix H.3

Repeat 1	Factor from trend line	retained growth per day	% loss per day	factor per generation	retained growth /generation
WT	-0,019	0,981	1,9	-0,04	0,96
520	-0,025	0,975	2,5	-0,05	0,95
580	-0,256	0,774	22,6	-0,51	0,60

Repeat 2	Factor from trend line	growth factor /day	% loss per day	factor per generation	retained growth /generation
WT	-0,01	0,990	1,0	-0,02	0,98
520	-0,008	0,992	0,8	-0,02	0,98
580	-0,258	0,773	22,7	-0,52	0,60

Repeat 3	Factor from trend line	growth factor /day	% loss per day	factor per generation	retained growth /generation
WT	-0,005	0,995	0,5	0,0	0,99
520	-0,283	0,754	24,6	-0,6	0,57
580	-0,224	0,799	20,1	-0,4	0,64

Repeat 4	Factor from trend line	growth factor /day	% loss per day	factor per generation	retained growth /generation
WT	0,0003	1,000	0,0	0,00	1,00
520	-0,027	0,973	2,7	-0,05	0,95
580	-0,247	0,781	21,9	-0,49	0,61

	growth factor /day					growth factor/generation*		
Repeat	WT	V520A	C580Y		Repeat	WT	V520A	C580Y
1	0,981	0,975	0,774		1	0,96	0,95	0,60
2	0,990	0,992	0,773		2	0,98	0,98	0,60
3	0,995	0,754	0,799		3	0,99	0,57	0,64
4	1,000	0,973	0,781		4	1,00	0,95	0,61
average	**0,992**	**0,924**	**0,782**		average	**0,983**	**0,863**	**0,611**
sd	0,007	0,098	0,011		sd	0,014	0,171	0,017
fitness cost/day	0,008	0,076	0,218		*assumption: 1 generation=2 days			
						fitness cost per generation		
					Repeat	WT	V520A	C580Y
					1	0,037	0,049	0,401
					2	0,020	0,016	0,403
					3	0,010	0,432	0,361
					4	-0,001	0,053	0,390
					average	**0,017**	**0,137**	**0,389**
					sd	0,014	0,171	0,017