

***N*-myristoyltransferase inhibitor binding mode
and phenotype in the malarial parasite.**

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Declaration

I, Anja Christine Schlott confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Malaria continues to be a significant cause of death and morbidity worldwide and there is a need for new antimalarial drugs with novel targets. As a potential target for drug development against malaria, the focus of this thesis is on *N*-myristoyltransferase (NMT), an enzyme that acylates a wide range of substrate proteins. The NMT substrates in *Plasmodium falciparum* include proteins that are important in essential processes including parasite signalling pathways, protein transport, and host cell invasion. Myristoylation facilitates the interaction of proteins with membranes, which may be strengthened by further lipidation, and inhibition of NMT results in incorrect protein localization and consequent disruption of function. The diverse roles of NMT substrates mean that NMT inhibition has a pleiotropic and severe impact on parasite development, growth and multiplication. To study the mechanism of action underlying NMT inhibition it is important to consider the function of proteins upstream and downstream of NMT. The phenotype of loss of myristoylation on particular substrates in isolation, through genetic manipulation of the N-terminal glycine, gives insights in the essentiality of this mostly co-translational modification.

Because there is an increasing threat of resistance to all front-line drugs against malaria, it is important to study resistance development early in the drug development process. This could aid the selection of drug combinations and impede rapid evolution of parasite resistance. This thesis describes the first parasites resistance mechanism to a particular NMT inhibitor series and shows that resistance in an *in vitro* parasite growth assay is mediated by a single amino acid substitution in the NMT substrate binding pocket. The basis of resistance was validated and analysed with a structure-guided approach using crystallography, in combination with enzyme activity, stability and surface plasmon resonance-assays, allowing identification of another inhibitor series unaffected by this substitution.

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Abbreviations

2-BP	2-bromopalmitate
ABE	Acyl-biotin exchange chemistry
ACTs	artemisinin combination therapies
AHA	methionine surrogate azidohomoalanine
AK2	adenylate kinase 2
AMA1	apical membrane antigen 1
AmBic	Ammonium Bicarbonate
APH	acylated pleckstrin-homology domain containing protein
ARF	adenosine diphosphate ribosylation factor
ARL	ADP-ribosylation factor-like
ARO	armadillo-domain containing rhoptry protein
Az	probe containing an azide functional group
AzTB	azido-TAMRA-biotin captur
BRE	B recondition element
BSA	bovine serum albumin
CaNMT	Candida albicans NMT
Cas9	CRISPR-associated protein 9 nuclease
CDPK	calcium-dependent protein kinase
CDPK1	calcium-dependent protein kinase 1
CDPK4	calcium-dependent protein kinase 4
CHX	cycloheximide
CNO	copy number polymorphism
CNP	copy number polymorphism
CoA	coenzyme A
CP/UF	conserved protein / unknown function
CPM	7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin
Cre	Cre recombinase enzyme
CRISPR	clustered regulated interspaced protospacer repeat
CTR	CQ resistance transporter
CuAAC	copper-catalysed azide/alkyne cycloaddition
DDT	dichlorodiphenyltrichloroethane
DHFR	dihydrofolate reductase
DHODH	dihydroorotate dehydrogenase
DHPS	sulfadoxine inhibiting dihydropteroate synthase
DiCre	rapamycin-induced dimerization system
DMSO	dimethylsulfoxide
DSB	double strand break

DTT	dithiothreitol
EC₅₀	half maximal effective concentration
ECL	essential light chain
EDTA	ethylenediaminetetraacetic acid
eED2	translation elongation factor 2
eEF2	translation elongation factor 2
EMA	European Agency for the Evaluation of Medicinal products
EMP1	erythrocyte membrane protein 1
ER	endoplasmic reticulum
ER	endoplasmic reticulum
ESI	electrospray ionisation
ETC	electron transport chain
FACS	fluorescence-activated cell sorting
FDA	U.S. Food and Drug Administration
FDR	false discovery rate
G2A	Gly2 to Ala mutant
GAp40	gliding-associated protein 40
GAP45	glideosome-associated protein 45
GCH1	GTP cyclohydrolase I
GNATs	GCN5-related <i>N</i> -acetyltransferases
GO	Gene Ontology
GPI	glycosylphosphatidylinositol
GRASP1	golgi re-assembly stacking protein 1
h	hours
h PI	hours post invasion
HA	hydroxylamine
HbS	sickle cell anaemia
HCD	high-energy collision dissociation
HPLC	high pressure liquid chromatography
HR	homologous recombination
HsNMT	Homo sapiens <i>N</i> -myristoyltransferase
IAA	Iodoacetamide
IC₅₀	half maximal inhibitory concentration
IFA	immunofluorescence assay
IMC	inner membrane complex
iRBC	infected RBCs
IRS	indoor residual insecticide spraying
ISP1	IMC sub-compartment proteins 1
ISP3	IMC sub-compartment proteins 3

ITNs	insecticide treated bed nets
K_i	inhibition constant
K_m	Michaelis-Menten constant
LC	liquid chromatography
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LFQ	label free quantification
m/z	mass-to-charge ratio
MDR1	multidrug resistance-1 transporter gene
MeOH	methanol
Met	methionine
MetAP	methionine aminopeptidase
MFI	median fluorescence intensity
MFS	malaria freezing solution
MMV	Medicine for Malaria Venture
MoA	mechanism of action
mPEG-Mal	methoxy(polyethylene glycol)-maleimide
MS	mass spectrometry
MSP	merozoite surface protein
MTCC	metabolic tagging with click chemistry
MTIP	myosin A tail domain interacting protein
MTS	malaria thawing solution
Myr-CoA	myristoyl coenzyme A
NDH2	NADH dehydrogenase
NDR	nucleosome-depleted regions
NEM	N-ethylmaleimide
NHEJ	non-homologous end joining
NMT	<i>N</i> -myristoyltransferase
NMTi	NMT inhibitor(s)
PAT	palmitoyl acyl transferase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDD	phenotypic drug development
PEG	polyethylene glycol
Pf	<i>Plasmodium falciparum</i>
PFA	paraformaldehyde
PfEMP1	erythrocyte membrane protein 1
PfNMT	<i>Plasmodium falciparum N</i> -myristoyltransferase
PfPI3K	phosphatidylinositol-3-kinase

PGH1	P-glycoprotein homolog 1
PI3P	phosphatidylinositol-3-phosphate
Pk	Plasmodium knowlesi
PK	pyruvate kinase
PKAr	protein kinase A regulatory subunit
PKB	protein kinase B
PKG	cGMP-dependent protein kinase
PKIP	Raf kinase inhibitor protein
PLC	Phospholipase C
PPM	metal-dependent protein phosphatases
PPM	parasite plasma membrane
PPM	Parasite plasma membrane
PTM	post-translational modification
PV	parasitophorous vacuole
Pv	Plasmodium vivax
PVM	Parasitophorous vacuole membrane
PvNMT	Plasmodium vivax <i>N</i> -myristoyltransferase
RAPA	rapamycin
RBC	red blood cell
RON2	rhoptry neck protein 2
RON4	rhoptry neck protein 4
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sgRNA	single guide RNA
SIT	sterile insect technique
SM1	salivary gland and midgut peptide 1
SrtA	<i>Staphylococcus aureus</i> Sortase A
STAGE-tips	stop-and-go extraction tips
TAMRA	tetramethylrhodamine
TBP	TATA-binding protein
TBTA	tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TC₅₀	half maximal tagging concentration
TCEP	tris(2-carboxyethyl)phosphine
TDD	target-based drug development
TEAB	triethylammonium bicarbonate
TFA	trifluoroacetic acid
TMT	tandem mass tag
Tris	tris(hydroxymethyl)aminomethane

TRR	tetratricopeptide-repeat protein
TSS	transcription start sites
TVN	tubulovesicular network
UPR	ubiquitin-proteasome system
V_{max}	maximum velocity
VPN	vacuolar protein sorting-associated protein
WB	Western blot
WHO	World Health Organisation
WR	WR99210
WT	wild type
Y_n	probe containing an alkyne functional group
Y_nC₁₂	tetradec-13-ynoic acid
Y_nC₁₆	heptadec-17-ynoic acid
Y_nTB	alkynyl-TAMRA-biotin capture reagent

Chapter 1. Introduction

1.1 Malaria is caused by apicomplexan parasites of the genus *Plasmodium*

Malaria, caused by the obligate intracellular apicomplexan parasites of the genus *Plasmodium* is spread by the bite of a female *Anopheles* mosquito. There are five species infecting humans: *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. malariae* and *P. ovale*. The latter is known to consist of two distinct subspecies: *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* (Sutherland et al. 2010). *P. falciparum* (Trager & Jensen 1976) and *P. knowlesi* (Moon et al. 2013) are the only two species where long term *in vitro* culturing methods exists, facilitating immensely the acquisition of knowledge on their biology. The *Apicomplexan* phylum is a group of unicellular eukaryotes which have a characteristic four-membrane bound cellular plastid called the apicoplast (McFadden et al. 1996). The only exception is the parasite *Cryptosporidium* which has lost this organelle through the course of evolution (Zhu et al. 2000). The apicoplast is thought to have arisen from a symbiotic alga that lost its photosynthetic properties. The closest known living descendant of such a symbiotic ancestor is the protist *Chromera velia*, which has a photosynthetic plastid bounded by four membranes (Moore et al. 2008).

1.2 The health burden of malaria

Malaria is still one of the world's most devastating infectious diseases, with little progress being made in reducing the global malarial cases between 2015-2017. There has even been an increase in malaria cases in the 10 highest burden countries. The number of clinical cases per year is estimated to be nearly a quarter billion leading to over half a million deaths (World Health Organization 2018b). Malaria is still present in over 80 countries, with 92% of all cases in the African Region followed by 5% in the South-East Asia Region and 2% in the Eastern Mediterranean Region (World Health Organization 2018b). *P. falciparum* — the most virulent and deadly species — accounts for 99.7% of the estimated clinical cases in 2017 in the African Region while *P. vivax* predominates in the American Region with 74.1% of clinical cases (World Health Organization 2018b). It is now appreciated that

there is a certain degree of underestimation of infections caused by the other human-infecting *Plasmodium* spp. This is mainly due to the difficulties in distinguishing *Plasmodium* species by microscopy, which is still the diagnostic gold standard for most malaria endemic countries (E. Baum et al. 2016; Doderer-Lang et al. 2014). Misdiagnosis is especially true for cases where species exists sympatrically with *P. falciparum* (Mayxay et al. 2004). Both *P. vivax* and *P. knowlesi* can cause severe disease and death while *P. malariae* and *P. ovale* have been increasingly found in complex mixed infections with *P. falciparum* and *P. vivax* and show important biological interactions with them (I. Mueller et al. 2007).

Clinical symptoms of malaria only result from the asexual blood stages of the infection (see section 1.3) causing symptoms such as fever, chills, nausea, vomiting, headache and diarrhoea in uncomplicated malaria. Severe malaria is more common in individuals with undeveloped or reduced immune protection (e.g. children or individuals with co-infections with other pathogens including bacteraemia and gastrointestinal helminths). It encompasses three often overlapping syndromes: severe anaemia, cerebral malaria and metabolic acidosis (L. H. Miller, Ackerman, Su & Wellems 2013b). Life-threatening severe forms of malaria are most often caused by *P. falciparum* but can also be caused by *P. knowlesi* and *P. vivax*. Severe anaemia results from haemolysis of erythrocytes and dysregulation of haematopoiesis (White 2018). Cerebral malaria is manifest as a coma with seizures often accompanied by respiratory distress and metabolic acidosis, and accounts for up to 20% of malaria-associated deaths (L. H. Miller, Ackerman, Su & Wellems 2013a). A major source of pathology during a severe malaria infection is due to sequestration of infected erythrocytes in deep tissue by binding to vascular endothelium and prevention of parasite clearance by the spleen. The binding of these cells is mediated by erythrocyte membrane protein 1 (PfEMP1), exported onto the surface of the infected red blood cell (RBC), and which binds to the endothelial surface proteins such as cluster of differentiation (CD) 36, intracellular adhesion molecule-1 (ICAM-1) and endothelial protein C receptor (EPCR) (Tuikue Ndam et al. 2017). PfEMP1 proteins are encoded by a repertoire of over 60 *var* genes (Su et al. 1995), from which one variety is expressed at a time and expression can be switched between them. This leads to antigenic variation and allows the parasite to evade the immune system. The sequestration of infected RBC damages endothelial cells and disrupts blood

flow, which causes tissue acidosis and lactic acid hypoxia, in turn leading to organ-specific syndromes such as cerebral or placental malaria (L. H. Miller, Ackerman, Su & Wellem's 2013a).

There are multiple factors which make effective control of the disease difficult. This includes the highly complex life cycle of the parasite (see section 1.3), increasing resistance to all frontline antimalarial drugs (see section 1.6.3) and the high polymorphism of the parasite which may affect the efficacy of a vaccine. Furthermore, increased human mobility (Askling et al. 2012) as well as climate change (Caminade et al. 2014) are likely to increase the geographical range of transmission due to increasing temperature and human migration. There has also been constant evolutionary pressure on the human host. *Plasmodium* is an ancient human pathogen with *P. falciparum* believed to have evolved alongside humans in Africa (Escalante et al. 1995). *P. falciparum* is closely related to a malarial parasite of chimpanzees, *P. reichenowi*, both believed to have diverged from a lineage of bird malaria dating back around 130 million years (Escalante et al. 1995; Carter & Mendis 2002). Similarly, it was thought that *P. vivax* was derived from macaque-infecting *Plasmodium* species in South East Asia (Escalante et al. 2005). However, it was recently discovered that both *P. falciparum* and *P. vivax* have evolved from an ape-infecting parasite in Africa, with *P. falciparum* undergoing a cross-species transmission event from gorilla to human (Liu et al. 2010; Liu et al. 2014) and *P. vivax* emerging from an ancestral stock of parasites that infected gorillas, chimpanzees and humans in Africa (Culleton & Carter 2012). This could mean that western gorillas are able to act as reservoirs of *P. falciparum* (Liu et al. 2010). The long evolutionary history of malaria infection in humans led to the imprinting of multiple polymorphisms into the human genome, which lead to various degree of protection against the disease. This is known as the 'malarial hypothesis' as first stated in 1949 by JBS Haldane (Haldane 1949). One example of a haemoglobin-inherited disorder is sickle cell anaemia (HbS). HbS is caused by a single nucleotide mutation at codon six of the β -globin gene, coding for valine instead of glutamic acid, and deforming the RBC to a sickle-shape (Ingram 1959). This polymorphism can lead in its lethal homozygote form to sickle-cell disease while the nonlethal heterozygote form can lead to a 10-fold decrease in severe malarial risk (Ackerman et al. 2005; Hill et al. 1991). Other mechanisms of protection involve other haemoglobinopathies such as

α - and β -thalassemia as well as two other haemoglobin structural variants HbC and HbE. Furthermore, it includes erythrocyte polymorphisms (Duffy blood group and ovalocytosis), immunogenic variants (e.g. complement receptor 1) and enzymopathies (pyruvate kinase (PK) and glucose-6-phosphate dehydrogenase (G6PD) deficiencies) (López et al. 2010).

Methods of malaria control discussed in section 1.6 including insecticide spraying, antimalarial drugs and bed nets, have resulted in a marked decrease in cases over recent years, encouraging malaria eradication as an end goal in the global health agenda. There are 46 countries now approaching elimination of malaria with 26 countries reporting less than 100 indigenous cases (World Health Organization 2018b). However, to achieve this goal significant obstacles need to be overcome. These include emerging drug and insecticide resistance, economic instability and underdevelopment of many countries where malaria is most widespread, increasing human mobility that creates a constant potential for malaria importation, still inadequate tools to tackle the dormant hypnozoite form that results in relapsing infections of *P. vivax*, and the lack of an efficacious vaccine (Battle et al. 2014; Feachem et al. 2010).

1.3 The *Plasmodium* life cycle

Plasmodium spp. have a very complex lifecycle alternating between a vertebrate host and a mosquito vector (Figure 1.1). Like most other Apicomplexan parasites, *Plasmodium* spp. are predominantly intracellular with only very brief extracellular moments when parasites exit one host cell and enter the next. During this brief moment, parasites have maximum exposure to the host immune system and therefore extracellular forms are targets for immunity induced by many vaccine candidates (see section 1.6). Exposure to the host immune system also increases the selection pressure on the parasite, which has evolved ways to circumvent it and that may cause some of the most virulent manifestations of the disease through variation of *var* genes expressing PfEMP1 (see section 1.2).

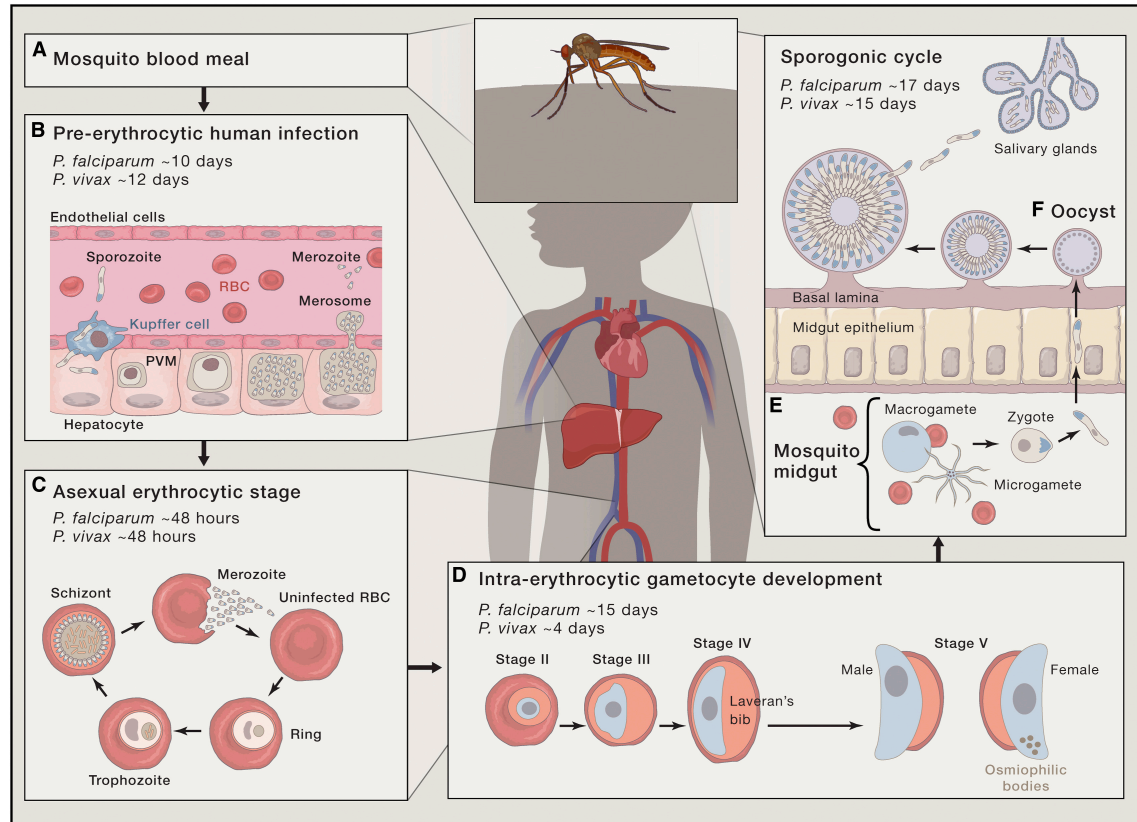


Figure 1.1 *Plasmodium* species life cycle. Comparison of *P. falciparum* and *P. vivax* in each stage. The life cycle is divided between vertebrate host and insect vector. A. Infection of a human host begins when a female *Anopheles* mosquito injects sporozoites into the skin. B. The pre-erythrocytic infection of *Plasmodium* starts with sporozoites migrating to the liver, where they exit the sinusoids through Kupffer or endothelial cells and invade hepatocytes. They form a parasitophorous vacuole membrane (PVM) and undergo schizogony generating daughter merozoites. C. During the asexual blood stage cycle, exponential parasite population growth occurs by repeated rounds of invasion, asynchronous replication and egress. D. A proportion of parasites will break from the asexual cycle and reprogram to undergo gametocytogenesis. E. The male and female gametocytes mediate transmission to the mosquito vector. They will undergo gametogenesis in the mosquito midgut, forming female macrogametes and exflagellated male microgametes. F. The micro- and macrogamete will fertilise generating a zygote. The zygote will in turn develop into an ookinete which embeds in the basal lamina of the midgut and develops further into an oocyst. The oocyst releases thousands of sporozoites that migrate to the mosquito salivary gland, prior to transfer to the vertebrate host. Taken from Cowman *et al.*, 2016.

Transmission between vertebrate hosts occurs through the bite of a female *Anopheles* mosquito, injecting ~10-100 (Medica & Sinnis 2005) motile haploid sporozoites from the salivary glands into the subcutaneous tissue rather than directly into the circulation (Ponnudurai *et al.* 1991; Sidjanski & Vanderberg 1997). The sporozoites migrate from the dermis towards the blood (~ 70%) and lymphatic systems (~30%) (Amino *et al.* 2006; Amino *et al.* 2008). Here they remain

extracellular for a short time until they are transported through the bloodstream to the liver where they invade hepatocytes through rupture of the plasma membrane followed by a rapid resealing mechanism (Mota et al. 2001). They migrate through several layers of endothelial cells, liver resident macrophages called Kupffer cells, and a few layers of hepatocytes (Tavares et al. 2013) until they reside in a hepatocyte where they replicate and differentiate into thousands of merozoites (Sturm et al. 2006). During this process sporozoites form an intrahepatic parasitophorous vacuole (PV) in which they replicate through schizogony, a process of repeated rounds of asexual replication of nuclear division without cytokinesis, to form a multinuclear syncytium called the hepatic schizont. This is followed by rupture of the PV and hepatocyte membrane in a process called egress, with release of hepatic merozoite-filled PV membrane-derived vesicles called merosomes, which protect the merozoites from the host immune system (Sturm et al. 2006). The merosomes disintegrate inside the pulmonary capillaries to release hepatic merozoites into the blood stream (Baer et al. 2007).

Merozoites enter erythrocytes to start the asexual replication cycle, resulting in the exponential proliferation of parasites. The lytic destruction of the erythrocyte during parasite egress results in some of the clinical manifestation of the disease (White et al. 2014) (see section 1.2). Egress is regulated by a cGMP-dependent protein kinase (PKG) together with calcium-dependent protein kinase 5 (CDPK5) (Absalon et al. 2018; Dvorin et al. 2010) which triggers a proteolytic cascade starting with the discharge of subtilisin-like serine protease SUB1 into the PV lumen (Collins, Hackett, et al. 2013; Yeoh et al. 2007). SUB1 is required for parasitophorous vacuole membrane (PVM) rupture, which occurs first, and cleaves and activates a putative cysteine protease called SERA6, a papain-like PV protein required for RBC membrane rupture (Collins et al. 2017; J. A. Thomas et al. 2018). SUB1 also cleaves merozoite surface protein 1 (MSP1), which interacts with the RBC cytoskeleton and is also involved in promoting egress (Silmon de Monerri et al. 2011; Das et al. 2015). Although merozoites appear to show motility after PVM rupture and just before egress, recent work has shown that the actomyosin motor is not essential for egress (Perrin et al. 2018). Invasion of the RBC involves merozoite attachment, RBC deformation, merozoite reorientation, and active entry through the force generated by the actomyosin motor (see section 1.5.1) into the RBC, followed by RBC resealing

and PVM formation (Cowman et al. 2017; Weiss et al. 2015). Initial contact between the merozoite and the erythrocyte can occur at any point on the merozoite surface followed by reorientation of the merozoite (Dvorak et al. 1975). *Plasmodium* has evolved multiple invasion pathways by an expansion of parasite invasion ligands, which may also help to evade the immune system. This includes the EBL and Rh protein families on the merozoite surface, which bind to different erythrocyte surface receptors (Tham et al. 2012; Weiss et al. 2015). After initial attachment and reorientation, anchoring is facilitated by a smaller member of the PfRH family called PfRH5 through a complex involving at least three other proteins, called CyRPA, RIPR and P113 (Reddy et al. 2015; Galaway et al. 2017; L. Chen et al. 2011; Knuepfer et al. 2019). This anchoring is followed by the formation of a 'moving junction' through the interaction of two merozoite proteins: apical membrane antigen 1 (AMA1) and rhoptry neck protein 2 (RON2) (Alexander et al. 2005; Tonkin et al. 2011). Both are secreted by specialised secretory organelles called micronemes (with secretion of AMA1 onto the merozoite surface) and rhoptries (with secretion of RON2 into the RBC plasma membrane). The 'moving junction' is passed around the merozoite driven by the actomyosin motor. The actomyosin motor is localised between the inner membrane complex (IMC) and the parasite plasma membrane (PPM). The IMC is a flattened vesicular structure lying just below the PPM, facilitating invasion in all invasive developmental forms (zoites) of *Plasmodium* and other apicomplexan parasites (Frénal et al. 2017). This creates a ~20- to 40-nm space between the IMC and the PPM, where the glideosome consisting of the actomyosin motor is localized (Frénal et al. 2010; Raibaud et al. 2001). The motor consists of a class XIV myosin (MyoA), short actin filaments, and associated myosin light chains called myosin A tail domain interacting protein (MTIP) and essential light chain (ELC), the integral membrane protein gliding-associated protein 50 (GAP50), GAP40, and GAP45 (Raibaud et al. 2001; Gaskins et al. 2004; Holder & Cowman 2006a; Green et al. 2017). PKG is essential for egress, and also phosphorylates key components of the glideosome including MyoA, GAP40 and GAP45 (Alam et al. 2015). The actomyosin motor is essential to generate the necessary force for the merozoite to invade the RBC (Green et al. 2017; Green et al. 2006). Invasion is completed as the 'moving junction' closes behind the merozoite leaving it enclosed within the PV. Successful invasion is quickly followed by a dramatic morphological change of the infected RBC from a biconcave disk to a spiky and uneven shape. This process is called

echinocytosis and lasts for about ten minutes. It is thought to be initiated by lipids released from the rhoptry organelles inducing a calcium-dependent osmotic water loss from the RBC (Weiss et al. 2015). Echinocytosis occur independent of the actomyosin motor, as it still occurs in a *GAP45* knock out (Δ *GAP45*) (Perrin et al. 2018) as well as in the presence of the actin polymerization inhibitor cytochalasin D (Weiss et al. 2015).

During asexual replication the parasite goes through four different stages: ring (1-14 hours (h)), trophozoite (14-30 h), schizont (30-48 h), and the individual haploid (1n) merozoite. During the trophozoite stage haemoglobin catabolism generates a waste product of insoluble ferrihaem that is polymerised and deposited as a crystalline form called hemozoin inside the food vacuole (Egan et al. 2002). At around 30 h post-invasion DNA replication starts — defining the onset of schizogony — which will generate a syncytium of 16–32 nuclei through asynchronous nuclear division without cytokinesis. Towards the end of schizogony the PPM wraps around the newly forming daughter merozoites, followed by rupture of the PVM and then of the RBC during egress (Wickham et al. 2003). During the erythrocyte cycle some parasites will undergo a developmental transformation to become gametocytes, to allow sexual transmission, during a process called gametocytogenesis. This is regulated by a transcription factor of the ApiAP2 family (Sinha et al., 2014). The maturation to male and female gametocyte takes 7–15 days (Bousema & Drakeley 2011). As the mature gametocytes are ingested by a feeding *Anopheles* mosquito, environmental stimuli such as reduced temperature, increase in pH (Billker et al. 1997) as well as the presence of the metabolite xanthurenic acid inside the mosquito midgut (Billker et al. 1998) induce gamete formation. Gametes egress from the RBC as flagellated male microgametes and female macrogametes, which will fuse inside the midgut to form the diploid (2n) zygote. Throughout the next 20 h the zygote will develop into an ookinete (4n) which is motile and able to move through the midgut to embed itself into the basal lamina, where it will mature into an oocyst. Through multiple rounds of DNA replication and mitosis, thousands of individual daughter sporozoites (1n) are generated following cytokinesis (Rosenberg & Rungsiwongse 1991). The fourth and final egress process is the exit of sporozoites from the oocyst, allowing them to travel to the mosquito salivary glands via the haemolymph and completion of the life cycle following injection into the next host.

1.4 Introduction to protein *N*-myristoylation

Post-translational modifications (PTMs) increase the diversity and complexity of a proteome by the incorporation and attachment of molecular motifs not encoded in the protein sequence. This includes, but is not limited to, the attachment of other proteins such as ubiquitin, the internal modifications of proteins through disulphide bond formation and proteolysis, the modifications of protein side chains through glycosylation, phosphorylation and palmitoylation, and N-terminal modification through acetylation and myristoylation.

A new drug target against malaria is the enzyme *N*-myristoyltransferase (NMT), which is ubiquitous in eukaryotes and is involved in PTM of proteins. The enzyme was first discovered in *Saccharomyces cerevisiae* (Towler et al. 1987) in 1987 but was not described in *P. falciparum* NMT (PfNMT) until 2000 by the Holder lab (Gunaratne et al. 2000). In contrast to *Plasmodium spp.* and other protozoa, all metazoa encode two NMT isoforms, for example human cells have NMT1 and NMT2 (Giang & Cravatt 1998). NMT has been shown to be essential for the survival of fungi, protozoa including *Leishmania major* (Tate et al. 2013; Wright et al. 2015), *Trypanosoma brucei* (Frearson et al. 2010), *Trypanosoma cruzi* (Herrera et al. 2016), *Toxoplasma gondii* (Alonso et al. 2019), and *Plasmodium falciparum* (Wright et al. 2014), as well as insects such as *Drosophila melanogaster* (Ntwasa et al. 2001), and plants such as *Arabidopsis thaliana* (Boisson et al. 2003). Furthermore the enzyme has been shown to be important in signalling pathways during the development of mice (Yang et al. 2005) and *C. elegans* (Tang & Han 2017), as well as being studied as a drug target against cancer (Thinon et al. 2016) and the common cold virus (Mousnier et al. 2018).

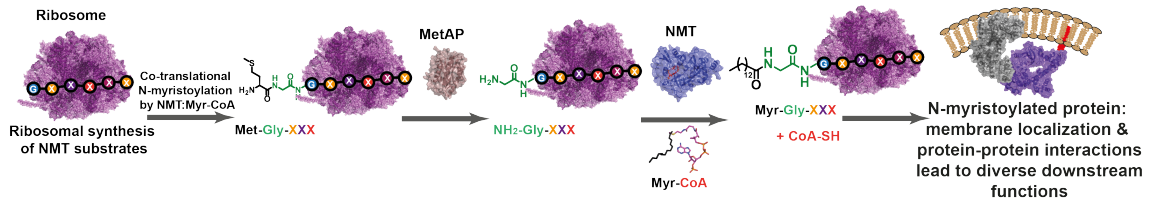


Figure 1.2 The mechanism of *N*-myristoylation. *N*-myristoylation is catalysed by the acyl transferase, *N*-myristoyl transferase (NMT), which attaches myristic acid to the N-terminal glycine of specific protein substrates. The modification is mostly co-translational following removal of the initiator methionine by methionine aminopeptidase (MetAP). The reaction catalysed by NMT follows an ordered Bi-Bi double displacement mechanism with Myr-CoA binding inducing a conformational change in NMT to allow substrate engagement. This is followed by transfer of the myristic acid to the substrate by nucleophilic substitution. Myristoylation promotes substrate localization to membrane, protein-protein interactions and protein stability. Figure taken from Schlott et al., 2018.

NMT catalyses the transfer of the 14-carbon saturated fatty acid myristate (C:14 tetradecanoic acid) from myristoyl coenzyme A (Myr-CoA) to the N-terminal glycine residue of specific substrates (Wright et al. 2010) (Figure 1.2). NMT belongs to a large enzyme family of GCN5-related *N*-acetyltransferases (GNATs) catalysing the transfer of an acyl moiety from a donor acyl-coenzyme A (CoA) to a diverse range of acceptor substrates (Ud-Din et al. 2016). *N*-myristoylation, although generally referred to as a PTM, is mostly a co-translational process, following the removal of the initiator methionine by methionine aminopeptidases (Wilcox et al. 1987). PTM through myristoylation has also been identified in human cells following the exposure of an internal glycine after caspase-mediated cleavage of proteins during the apoptotic cascade (Thinon et al. 2014; Thinon et al. 2016). Bioinformatic analyses using two main prediction tools: MYR predictor (Maurer-Stroh, B. Eisenhaber & F. Eisenhaber 2002a) and Myristoylator (Bologna et al. 2004) suggest that up to 2% of the 5400 proteins in *P. falciparum* are myristoylated.

1.4.1 Catalytic mechanism and structure

The first crystal structure of NMT was that of *Candida albicans* NMT (CaNMT) published in 1998 (Sternglanz & H. Schindelin 1999). The study showed that the enzyme is monomeric and has a compact globular structure. Since then many NMT structures have been published with the first *P. vivax* (PvNMT) structure being solved in 2012 (Goncalves, Brannigan, Whalley, et al. 2012). A homology modelled *P. falciparum* NMT (PfNMT) structure was published in 2015 based on *P. vivax*

structures (Paul et al. 2015), but no *P. falciparum* crystal structure has been published to date. The transfer of myristic acid from Myr-CoA to the N-terminal glycine catalysed through NMT follows an ordered Bi-Bi catalytic mechanism (Rudnick et al. 1991) with the binding of Myr-CoA to the apo-enzyme inducing a conformational change that allows the peptide substrate to bind. The C-terminal carboxyl residue (Leu in PfNMT) acts as a base at physiological pH and is probably responsible for deprotonating the amine group of the N-terminal glycine. This is now much more reactive in its deprotonated form leading to a nucleophilic attack of the thioester bond between the coenzyme bound to myristic acid. The acyl group is transferred from Myr-CoA to the N-terminal glycine residue via an amide bond. This releases the CoA product followed by the release of the myristoylated substrate. The conformational changes are not yet fully understood and there is evidence for a water-mediated deprotonation by HsNMT rather than by the C-terminal carboxylate directly (personal communication with Edward Tate). Experiments have also suggested that the rate-determining step is not the chemical transformation, but the release of the myristoylated peptide (Thalia A Farazi et al. 2000).

1.4.2 NMT substrate specificity

NMT has two substrates: Myr-CoA and the peptide substrate. While the substrate binding pocket varies considerable among different species, the Myr-CoA binding pocket is very conserved across all NMT enzymes (Heuckeroth et al. 1988; Kishore et al. 1993). The chain length of myristic acid compared to other acyl chains is important for NMT recognition but one methylene shorter (13-C) or longer (15-C) can still be tolerated (Rudnick et al. 1992). Although this recognition is not very efficient, the high concentration of the 16-C-palmitoyl-CoA inside the cell could serve as a competitive inhibitor to regulate NMT activity (Bhatnagar et al. 1997). The general consensus sequence for *N*-myristoylation of the NMT substrate occurs around the first 10 (even up to 17) amino acids: Met-Gly-X-X-X-Ser/Thr-X-X-X-X (Figure 1.3) (Maurer-Stroh, B. Eisenhaber & F. Eisenhaber 2002b). The recognition sequence is broadly conserved with Gly2 being absolutely required and position six being a preferred serine or other polar residue (Wright et al. 2010). Residues 1-6 are bound in the enzyme active site pocket, residues 7-10 (small and polar) interact with the

surface of NMT around the catalytic pocket and the hydrophilic linker of residues 11-17 are presumably involved in preventing the N-terminal sequence region from folding, which would prevent recognition by NMT (Maurer-Stroh, B. Eisenhaber & F. Eisenhaber 2002b).

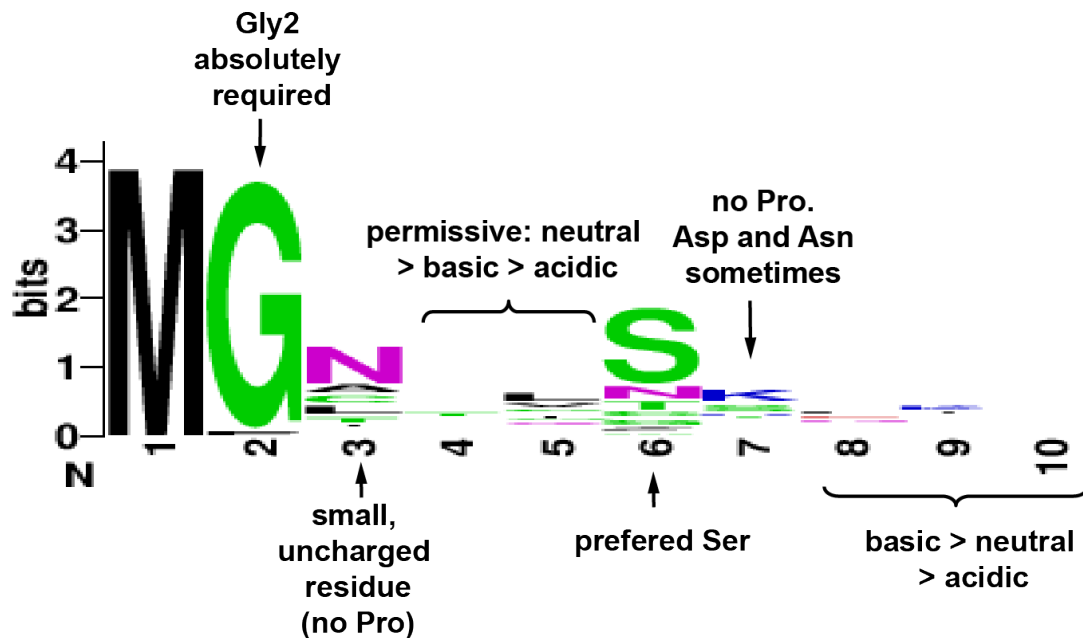


Figure 1.3 Summary of peptide substrate specificity of *P. falciparum* N-myristoyltransferase (PfNMT).

The first 10 amino acids of the 32 identified substrates from Wright *et al.*, 2014 were used to generating a consensus myristoylation sequence motif using the tool available at: <https://weblogo.berkeley.edu/logo.cgi>. Residues are numbered from the leader methionine, which must be cleaved by methionine aminopeptidase prior to myristoylation. Description adapted from Wright *et al.*, 2010.

1.4.3 NMT regulation

As previously mentioned *Plasmodium spp.* and other protozoa only contain one NMT isoform while all metazoa encode two NMT isoforms (e.g. human cells encoding Homo sapiens N-myristoyltransferase 1 and 2 (HsNMT1 and HsNMT2)), which have 75% sequence identity (Giang & Cravatt 1998) (Table 1.1). *P. falciparum* NMT shares 48% and 49% identity with HsNMT1 and HsNMT2. HsNMT 1 and 2 diverge most at their N-terminal sequence (Giang & Cravatt 1998) which is thought to be involved in targeting NMT to a particular subcellular location such as the ribosome (Glover *et al.* 1997; Wilcox *et al.* 1987).

% identity	LdNMT	TbNMT	PfNMT	PvNMT	HsNMT1	HsNMT2
LdNMT	100	57	41.33	42.09	43.44	44.99
TbNMT	57	100	39.69	40.98	45.74	49.1
PfNMT	41.33	39.69	100	80.49	48.28	49.01
PvNMT	42.09	40.98	80.49	100	51.48	50
HsNMT1	43.44	45.74	48.28	51.48	100	75.31
HsNMT2	44.99	49.1	49.01	50	75.31	100

Table 1.1 Comparison of amino acid identity of *N*-myristoyltransferase (NMT) orthologues.

The percentage identity of NMT amino acid sequences from different organisms determined with cluster omega, with NCBI accession number given in brackets: *Plasmodium falciparum* NMT (PfNMT) (AAF18461), *Leishmania donovani* NMT (LdNMT) (CBG76455), *Trypanosoma brucei* NMT (TbNMT) (RHW69826), *Homo sapiens* NMT 1 (HsNMT 1) (AAC09294), HsNMT2 (AAH06376), *Plasmodium vivax* NMT (PvNMT) (EDL47099).

In 2002 this N-terminal basic amino acid region termed the “K box” in HsNMT1 and HsNMT2 was shown to be sufficient and essential for binding the ribosome but it is not conserved in other NMTs (Takamune et al. 2010). There are few data showing regulation of NMT in *P. falciparum*, especially *in vivo*. PfNMT is generally constitutively expressed in all stages and mostly cytoplasmic although some portion is associated with membrane proteins, as also described for *Leishmania major* NMT (Price et al. 2003). *Trypanosoma cruzi* NMT, for example, has also been shown to be expressed in all stages of the parasite and was localized to the endoplasmic reticulum (ER) (Herrera et al. 2016). As previously mentioned palmitoyl-CoA, being only two methylenes longer, can serve as a poor substrate and because of its higher concentration as a competitive inhibitor for NMT (Bhatnagar et al. 1997) (see section 1.4.2). It is therefore thought that it needs to be made unavailable to NMT by sequestration by acyl carrier proteins (Bhatnagar et al. 1994). From the regulation of *S. cerevisiae* NMT it is known that palmitoyl acyltransferase, the enzyme that attaches a palmitoyl group to a cysteine -SH group as a thioester on its substrate, is localized at the membrane (Johnson, Bhatnagar, et al. 1994). It is thought that NMT modifies its substrate first through the co-translational process. This increases affinity of this substrates for membranes, which in turn allows them to be palmitoylated, resulting in permanent anchoring of these proteins and giving them similar localisation as the palmitoyl transferases (Berthiaume & Resh 1995; Wright et al. 2010). Furthermore the regulation of availability of Myr-CoA through acyl-CoA

synthetase and fatty acid synthetase has been studied in *S. cerevisiae* (Johnson, Knoll, et al. 1994).

1.4.4 Dual acylation of *N*-myristoylated proteins and its regulation

Myristoylated proteins are mostly localised at a membrane with myristic acid interacting with the lipid bilayer and increases the affinity for the membranes (Wright et al. 2010; Schlott et al. 2018). The Gibbs free energy released by interaction of the myristoyl group with the lipid bilayer of 8 kcal/mol or a dissociation constant of 10^{-4} M, is thought to be insufficient to tightly anchor most substrates (Peitzsch & McLaughlin 1993). This creates the necessity for a second signal of the protein to complement myristoylation, such as a second acyl group near the N-terminus. This led to be 'two signal membrane binding model' where a strong interaction can only be generated when a second modification complements myristoylation (Resh 1994). Therefore, many myristoylated proteins have a second acyl group near the N-terminus, such as an S-acylation via thioesterification. But they can also have a polybasic cluster of amino acids interacting with the membrane (e.g. interaction of positively charged SH4 domain of human NMT substrate c-Src with negatively charged lipids in the membrane (Diana Murray et al. 1998)) or a protein domain can interact with other membrane-bound proteins through protein-protein interactions (Resh 2006). Several studies using point mutation analysis of the N-terminus of dual acetylated proteins have shown that the second signal is essential (Cabrera et al. 2012; Wetzel et al. 2015; Ridzuan et al. 2012). Compared to myristoylation, which is mostly co-translational, palmitoylation is a reversible post-translational modification where palmitic acid is linked to cysteine residues via a thioester bond, catalysed by palmitoyl acyl transferase (PAT) (Resh 1999). Plasmodium encode 12 DHHC palmitoyltransferase (DHHC1-12) (Frénal et al. 2013). Known *P. falciparum* NMT substrates (Wright et al. 2014) (see section 1.5) were searched for the presence of known palmitoylation published in the palmitoyl proteome (Jones et al. 2012) or published in Cabrera et al., 2012. Eleven proteins were identified as likely dual acylated with both the N- and C-termini (e.g. GAP45) being palmitoylated (Ridzuan et al. 2012) or just one cysteine being palmitoylated close to the N-terminus (e.g. armadillo-domain containing rhoptry protein, ARO) (Cabrera et al. 2012) (Table 1.2).

Cabrera et al., identified 18 proteins predicted to be dual acylated within the first 20 amino acids, while genome analysis suggests that there are up to 25 proteins that can be both myristoylated and palmitoylated at the N-terminus (Cabrera et al. 2012).

Protein ID	Protein name	Jones et al., 2012 (palmitoylation shown experimentally)	Calebra et al., 2012 (palmitoylation prediction)	other publications
PF3D7_1020900	ADP-ribosylation factor 1 (ARF1)	x		Calebra et al., 2012
PF3D7_0414900	Armadillo-domain containing rhopty protein (ARO)		x	
PF3D7_0217500	Calcium-dependent protein kinase 1 (CDPK1)	x	x	Rees-Channer et al., 2006
PF3D7_1137300	CLPTM1 domain containing	x		
PF3D7_1237700	Conserved protein unknown function	x		
PF3D7_1222700	Glideosome-associated protein 45 (GAP45)	x	x	Möskes et al., 2004
PF3D7_1460600	IMC protein 3 (ISP3)	x	x	
PF3D7_1011000	IMC protein 1 (ISP1)	x	x	
PF3D7_0810300	Metal-dependent Protein phosphatase 5 (PPM5)	x	x	
PF3D7_1310600	Ras-related protein Rab-5B, GTPase (Rab-5b)	x	x	
PF3D7_0816200	Vacuolar protein-sorting protein (VPS2)	x	x	
PF3D7_1362400	Calpain	x	x	Russo et al., 2009
PF3D7_1309200	Metal-dependent Protein phosphatase 6 (PPM6)	x *	x	
PF3D7_0913800	Conserved protein unknown function		x	
PF3D7_1223100	PKAr		x	
PF3D7_1437400	Pantothenate kinase		x	
PF3D7_0920500	ADP-R factor-like		x	
PF3D7_0816900	Adenylate kinase 2 (AK2)		x	

Table 1.2 List of *N*-myristoyltransferase substrates in *P. falciparum* which are likely to be dual acylated, with at least one cysteine palmitoylation site.

The two main papers showed evidence for palmitoylation experimentally (Jones et al., 2012) and through predictions (Calebra et al., 2012). Further publications for experimental evidence are given. Proteins identified by the YnPal pull down but not statistically enriched by the acyl-biotin exchange methods used in Jones et al., 2012 are indicated with an asterisk (*).

The two-signal binding model provides a mechanism by which the membrane binding strength might be regulated by a 'myristoyl-switch' as studied for the NMT substrate ARF1 (see section 1.5.2). Ligand binding induces a conformational change, flipping the myristoyl group from an exposed to a buried conformation by hiding it in a hydrophobic binding pocket of ARF1, allowing it to associated with and dissociate from the membrane as part of its function (Wright et al. 2010).

1.5 Location and function of NMT substrates during the erythrocyte life cycle

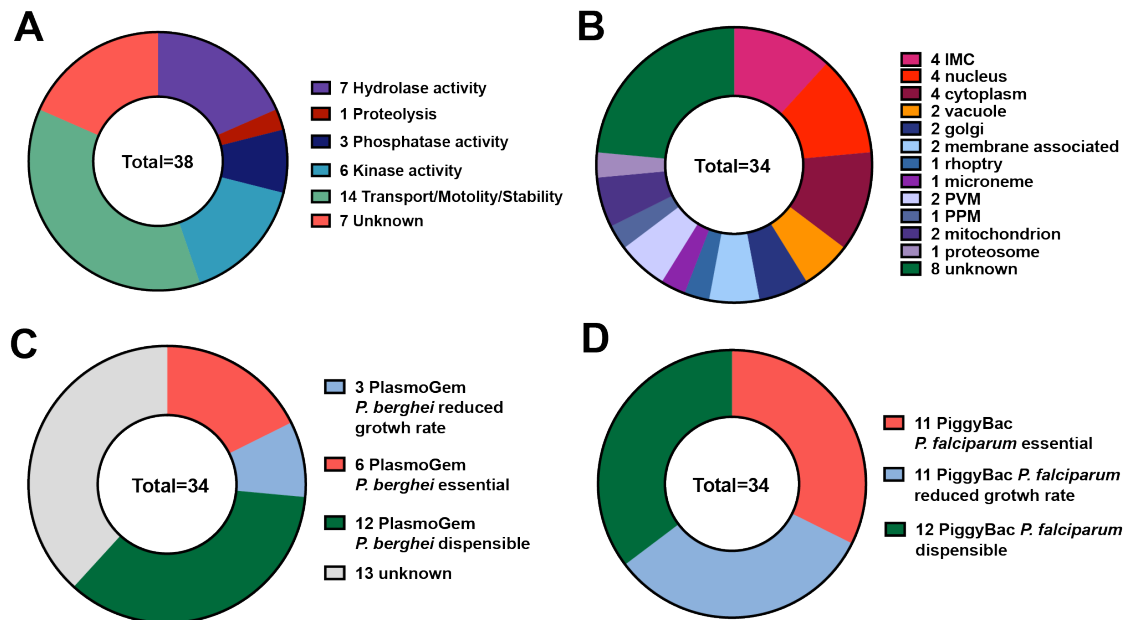


Figure 1.4 NMT substrate analysis for function, localisation and essentiality.

Analysis of 32 known *N*-myristoylated proteins identified by Wright *et al.*, 2014 (including AK2 and Calpain) A. Putative function with some substrates receiving multiple assignments. B. Putative localisation. For A and B: assigned by using PlasmoDB, GO Retriever, and literature searches. C. Putative essentiality for the erythrocyte life cycle of *P. berghei* NMT substrates established through a gene knock out screen (PlasmoGem Bushell *et al.*, 2017 and Schwach *et al.*, 2015). D. Putative essentiality for the erythrocyte life cycle of *P. falciparum* NMT substrates established through a piggyBac insertional mutagenesis screen (Zhang *et al.*, 2018). Figure adapted from Schlott *et al.*, 2018. For detailed categorisation of each substrate see table 7.1, 7.2, and 7.3 in the appendix.

NMT substrates are involved in a diverse range of biochemical and cellular pathways including: secretion, protein transport, protein homeostasis, ion channel regulation, the formation of the IMC, as well as in parasite motility and development (Wright *et al.* 2014). Their known biological functions range from kinase to phosphatase and hydrolase activities, and stabilizing the parasite motor complex that drives invasion (Figure 1.4 A, Figure 1.5) (Schlott *et al.* 2018). In addition to being localized to membranes including the PPM and IMC, substrates are also targeted to various organelles such as the rhoptries and micronemes (Figure 1.4 B, Figure 1.5) (Schlott *et al.* 2018). About one third of the NMT substrates identified by Wright *et al.*, have been shown to be essential in the recent *P. falciparum* piggyBac insertional mutagenesis screen (Zhang *et al.* 2018) as well as the *P. berghei* PlasmoGem knock

out screen (Schwach et al. 2015; Bushell *et al.*, 2017) (Figure 1.4 C, D). The method for the PlasmoGem Screen was established in Gomes et al., (Gomes et al., 2015). Both screens have their limitations and advantages with the *P. falciparum* screen only studying genes essential for blood stages, while the *P. berghei* screen also has the potential to inform about the essentiality of genes expressed in liver, gametocyte and mosquito stages if extended to the complete life cycle. Neither screen provided any further insight into the function of essential genes because the mutant parasites cannot be isolated. Taking the data from both screens together can only give an indication of the essentiality of a gene. It requires further single conditional gene disruption experiments to study the phenotype of those essential genes. The following summary of the function and localization of NMT substrates is an extended version of what was recently published in Schlott et al., 2018.

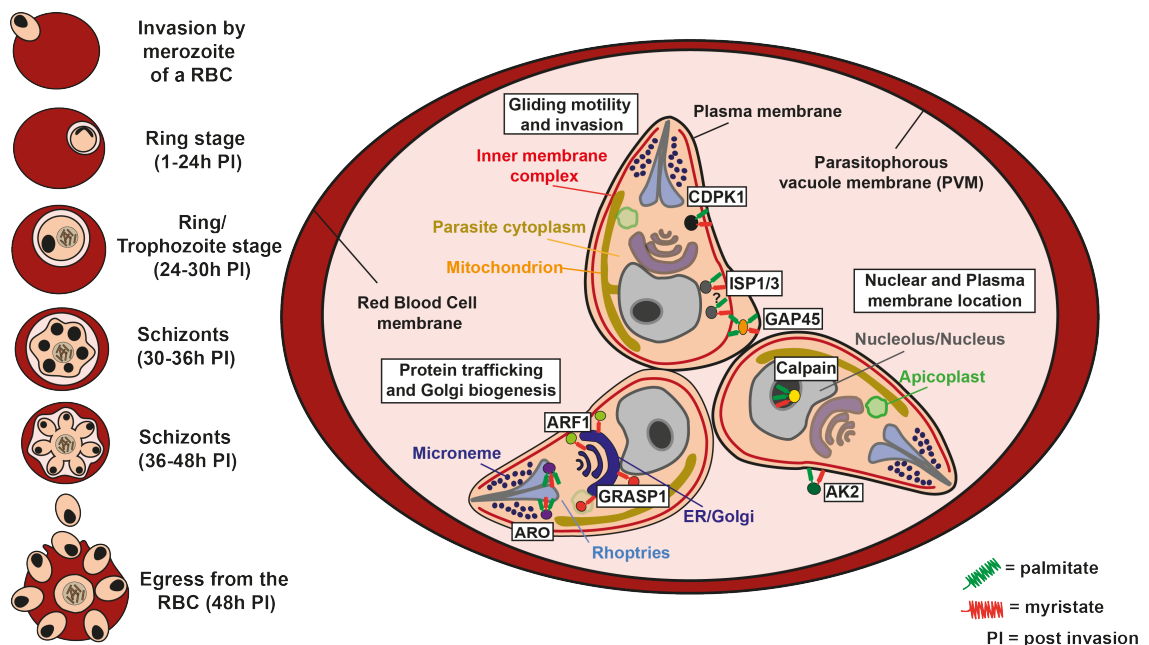


Figure 1.5 NMT substrates are localised at various membranes.

P. falciparum NMT substrates are involved in a variety of essential processes including host cell invasion, parasite gliding motility, protein trafficking and protein degradation. Inhibition of NMT leads to the mis-localisation and mis-function of these substrates, causing catastrophic and irreversible failure to continue through the life cycle. Figure taken from Schlott *et al.*, 2018.

1.5.1 NMT substrates and their function at the PPM and IMC

The two most studied NMT substrates in *P. falciparum* are calcium-dependent protein kinase 1 (CDPK1) and GAP45. Both proteins have been shown to be dual acylated with N-terminal myristoylation and N-terminal (and C-terminal in the case of

GAP45) S-palmitoylation (Möskes et al. 2004; Rees-Channer et al. 2006) (**Table 1.2**). CDPK1 requires *N*-myristoylation for localization to the PPM (Bansal et al. 2013; Green et al. 2008) (Figure 1.5), where it phosphorylates IMC proteins, including MTIP, Raf kinase inhibitor protein (RKIP) and GAP45 (Green et al. 2008; Kugelstadt et al. 2007). CDPK1 is likely non-essential in the asexual blood stage, at least in *P. berghei* (Schwach et al. 2015; Sebastian et al. 2012; Jebiwott et al. 2013), but was classified as essential in the recent *P. falciparum* high-throughput piggyBac transposon insertional mutagenesis screen (Zhang et al. 2018). Furthermore, in sexual stages attempted knockdown of CDPK1 prevented parasite transmission to mosquitoes and resulted in developmental arrest (Sebastian et al. 2012). GAP45 is encoded by a single exon, is anchored to the PPM via dual acylation of its N-terminus and to the IMC via acylation at its C-terminus, thus bridging the two membranes (Figure 1.5) (Ezougou et al. 2014; Ridzuan et al. 2012). In addition to the *N*-myristylation at glycine 2 and palmitoylation at cysteine residue 5 (Ridzuan et al. 2012) it has five cysteines at its C-terminus. C160 has been shown to be palmitoylated (Jones et al. 2012) while the remaining four cysteines are potential palmitoylation sites (C176, C178, C189, and C192). GAP45 also has eight *in vivo* phosphorylation sites: S89, S103, S107, S142, S149, S156, S158, and S198 (Treeck et al. 2011). S98 and S103 have been shown to be phosphorylated by CDPK1 (Ridzuan et al. 2012), S103 and S149 phosphorylation is being influenced by phospholipase C mediated calcium release (D. C. Thomas et al. 2012), S103 is phosphorylated by protein kinase B (PKB) (D. C. Thomas et al. 2012) and S149 and S159 phosphorylation has been shown to be PKG-dependent (Alam et al. 2015). GAP45 is thought to be involved in binding both MyoA and MTIP and stabilizing the actomyosin motor complex that drives merozoite invasion (J. Baum, Richard, Healer, Rug, Krnajski, Gilberger, Green, Holder & Cowman 2006b; Ridzuan et al. 2012; Perrin et al. 2018). Experiments with episomally expressed truncated GFP-fusion GAP45 showed that the first 30 N-terminal amino acids of GAP45 are sufficient to localize it to the PPM, whereas a truncated GAP45 lacking the N-terminal 30 amino acids localizes to the IMC due to the C-terminal S-palmitoylation (Ridzuan et al. 2012). This study did not address whether myristoylation of GAP45 is essential for its function and therefore for the viability of the parasite, as the endogenous GAP45 gene was still expressed. Very recently a conditional knockout of GAP45 showed that it is essential for invasion but not for parasite egress (Perrin et al. 2018).

In addition to GAP45 two additional NMT substrates are located at the IMC, called IMC sub-compartment proteins 1 and 3 (ISP1 and ISP3) (Poulin et al. 2013) (Figure 1.5). The roles of these proteins have been studied in *P. berghei* through reverse genetics and live cell imaging (Poulin et al. 2013). ISP1 has been shown to be essential in *P. berghei* (Poulin et al. 2013) as well as in the recent *P. falciparum* insertional mutagenesis screen (Zhang et al. 2018), whereas ISP3 has been found non-essential in *P. berghei* but having a slow growth rate when disrupted in the in *P. falciparum* screen (Zhang et al. 2018). The proteins are expressed in all invasive developmental stages, and due to their localization at the anterior apical end where IMC formation initiates, they are thought to be involved in IMC formation and organization together with GAP45, through the establishment of polarity (Poulin et al. 2013). Furthermore, work in *P. berghei* has shown that the absence of ISP3 alters the apical localization of ISP1 in invasive stages, indicating coordinated function of the two proteins (Poulin et al. 2013).

1.5.2 NMT substrates involved in transport and secretory organelle localisation

The largest functional group of NMT substrates is involved in transport (Figure 1.4 A). This includes the secretory pathway for exporting soluble proteins to the PV and proteins such as merozoite surface proteins including MSP1 to the PPM. Examples of NMT substrates involved in this process are amongst the most abundant *N*-myristoylated proteins identified by Wright *et al.* (Wright et al. 2014): ADP-ribosylation factor 1 (ARF1) and two ADP-ribosylation factor-like (ARL) proteins (PF3D7_0920500 and PF3D7_1034700). ARFs and ARLs are members of the Ras superfamily of low molecular weight GTP-binding proteins. Some ARFs lack the GTPase activity but are enhancers for cholera toxin ADP ribosyl-transferase activity when GTP is bound, as shown in other organisms (Kahn & Gilman 1984). ARLs on the other hand fail to activate cholera toxin but are able to hydrolyse GTP (Tamkun et al. 1991). ARF1 is localized at the Golgi complex and is involved in trafficking processes of the secretory pathway and vesicular biogenesis, as well as being involved in other membrane trafficking steps including those at the ER and the nuclear envelope in other organisms (Boman & Kahn 1995). Its function includes recruiting coat proteins to promote the assembly of a coating complex for cargo

secretion by vesicular budding and in determining cell structure by interacting with cytoskeletal factors. ARF1 has been co-localized with Golgi marker Golgi re-assembly stacking protein 1 (GRASP1) which itself is myristoylated in *P. falciparum* (Thavayogarajah et al. 2015). Due to its high homology across species, the functions in other organism are likely to be conserved in *P. falciparum* (Donaldson & Jackson 2011). The myristate of human ARF1 switches between a buried and an exposed state (the myristoyl-switch), allowing ARF1 to associate with and dissociate from membrane as part of its function (Wright et al. 2010) (see section 1.4.4).

Other NMT substrates associated with secretory organelles are localized to the membranes of micronemes and rhoptries. ARO is localized to rhoptries at the apical end of merozoites (Cabrera et al. 2012) (Figure 1.5) at both the rhoptry bulb and neck through analogy with *Toxoplasma gondii* (C. Mueller et al. 2013)). Analogy with TgARO may also indicate its involvement in rhoptry positioning to the apical end of the cell, where the rhoptries discharge their contents during host cell invasion (C. Mueller et al. 2016). An inducible *aro* gene knockout in *T. gondii* caused mislocalisation and cytosolic dispersion of the rhoptries, resulting in severe defects in invasion but no effect on intracellular growth or egress from the host cell (C. Mueller et al. 2016). A recently characterized protein now named acylated pleckstrin-homology domain-containing protein (APH) (PF3D7_0414600) was also found to be myristoylated (Wright et al. 2014). APH is homologous to a protein coded by a gene (TGME49_249970) found to be essential in a genome-wide CRISPR screen in *Toxoplasma* (Sidik et al. 2016) and gene modification resulted in a slow growth rate in both the *P. berghei* reverse genetic screen (Bushell et al., 2017; Schwach et al. 2015) and the *P. falciparum* mutagenesis screen (Zhang et al. 2018). In *Toxoplasma*, myristoylation (G2) and palmitoylation (C7) of APH is thought to be involved in localizing the protein to the outer surface of micronemes, where it is involved in sensing phosphatidic acid (PA), inducing microneme secretion (Bullen et al. 2016). In *Plasmodium*, APH has not been identified in the palmitoyl proteome (Jones et al. 2012) or predicted to be palmitoylated (Cabrera et al. 2012) but does contain a cysteine at position 9.

Other known NMT substrates also have predicted transport functions including Rab5b, a member of the Rab family of GTPases, GRASP1, vacuolar protein-sorting

protein 2 (VPS2; PF3D7_0816200), and vacuolar protein sorting-associated protein 46 (VPS46; PF3D7_0906100). GRASP1 is highly conserved at the N-terminus across most species with the myristoylation motif required for Golgi localisation in close proximity to the nucleus (Struck et al. 2005), where it is thought to be involved in maintaining Golgi integrity. The presence of two GRASP splicing variants has recently been reported leading to two different isoforms of which only GRASP1 is myristoylated whereas GRASP2 lacks myristoylation and instead contains a hydrophobic N-terminal sequence interacting with the Golgi membrane; its location resembles the distribution of GRASP1 but its structure may result in a stronger binding to the membrane (Struck et al. 2008). In the recent *P. falciparum* mutagenesis screen (Zhang et al. 2018) the gene has been found to be dispensable. Rab5b is both myristoylated and palmitoylated (Quevillon et al. 2003), and has been shown to localize to the food vacuole and PPM (Ezougou et al. 2014). It co-localizes with adenylate kinase 2 (AK2) (see section 1.5.4) and both may be transported to the tubulovesicular network (TVN) extending from the PVM through a membrane-trafficking pathway that is still largely unknown (Ebina et al. 2016). In addition to being myristoylated and palmitoylated, Rab5b may be prenylated on either of three internal cysteine residues (Gisselberg et al. 2017), and these lipidations may be important for its localisation to both the food vacuole and the PV (Howe et al. 2013). Both VPS proteins were identified as myristoylated (Wright et al. 2014) and may play a role in vesicle tethering, by homology with VPS proteins in other organisms (Cai et al. 2007). In *Plasmodium*, vesicular trafficking involving VPS proteins may also prepare parasites for the host cell invasion process (Tomavo et al. 2013). Both VPS proteins were found to be essential in the recent *P. falciparum* mutagenesis screen (Zhang et al. 2018).

1.5.3 NMT substrates with hydrolase activity

In addition to ARF1, the two ARF-like proteins and Rab5b, there are two additional NMT substrates with hydrolase activity, the alpha/beta hydrolases PF3D7_0805000 (S9B) and PF3D7_0403800 (S9C). Both belong to the S9 protein family conserved in all *Plasmodium* species, which comprises three members: S9A, S9B, and S9C. Only S9C and S9B have N-terminal catalytic domains and have been shown to be myristoylated (Wright et al. 2014), with S9C also being heavily phosphorylated

(Treeck et al. 2011; Lasonder et al. 2012; Pease et al. 2013). S9C showed a slow-growing phenotype in the *P. berghei* reverse genetic screen (Bushell *et al.*, 2017; Schwach et al. 2015) while it was identified as essential in the *P. falciparum* mutagenesis screen (Zhang et al. 2018). S9B on the other hand is likely not essential based on both screens.

The 26S proteasome regulatory subunit 4 (PRT2) (PF3D7_1008400) was also identified as an NMT substrate through the detection of its modified N-terminal peptide by mass spectrometry (Wright et al., 2014). The mammalian homologue, PSMC1, is important for the function of the proteasome in platelet formation and brain development (Rezvani et al. 2012; Shi et al. 2014). In *Plasmodium*, PRT2 is likely essential based on the result of the *P. berghei* gene knock out screen (Bushell *et al.*, 2017; Schwach et al. 2015) as well as the *P. falciparum* piggyBac insertion mutagenesis screen (Zhang et al. 2018). The proteasome is a known drug target in *Plasmodium* especially in the fight to overcome resistance to artemisinin by blocking its involvement in cell stress response, which is elevated in resistant parasites (H. Li et al. 2012; Tschan et al. 2013; H. Li et al. 2016) (see section 1.6.4). In addition to the eukaryotic 26S proteasome, *Plasmodium spp.* also possess a homologue of the prokaryotic proteasome called caseinolytic protease Q (ClpQ, PF3D7_1230400) also known as heat shock locus V (HslV) as well as a homologue of the cyanobacterial Clp protease (PfClpP, PF3D7_0307400), highlighting the importance of the degradation system for the survival of the parasite (Ng et al. 2017).

1.5.4 NMT substrates and their involvement in signalling pathways

NMT substrates with a role in phosphorylation/dephosphorylation switches in signalling pathways include calcium-dependent protein kinase 4 (CDPK4) and CDPK1. CDPKs are absent from animals and have only been found in plants, ciliates, and apicomplexan parasites. Their exact roles are not fully understood, but they may regulate intracellular calcium levels and cell cycle progression through their stage-specific expression (Holder et al. 2012). CDPK4 is thought to be dispensable in the asexual life cycle but essential for sexual reproduction and mosquito transmission in *P. berghei* (Billker et al. 2004). Here, CDPK4 has a role in activating replication in haploid microgametocytes, assembling the mitotic spindle by phosphorylating microtubule-associated proteins, as well as completing cytokinesis

by activating the motility of the male gamete (Fang et al. 2017). For most of these functions myristoylation is thought to be essential, providing an indication of its importance in sexual stages. A second smaller isoform of CDPK4 that is not myristoylated has been identified and is thought to be essential to complete late gametogenesis, while the longer myristoylated isoform may initiate the first round of DNA replication (Fang et al. 2017). The myristoylated cAMP-dependent protein kinase A regulatory subunit (PKAr) (Haste et al. 2012; Wright et al. 2014) is the regulator of PKA, which is important in cell invasion during the asexual stages, in gametogenesis, as well as in merozoite formation inside hepatocytes, as reviewed in Wurtz *et al.* (Wurtz et al. 2011). Because of its importance for many aspects of parasite biology PKA represents an attractive drug target (Haste et al. 2012), although it may be difficult to obtain selectivity over the host enzyme. Two metallo-dependent protein phosphatases (PPM) called PPM2 and PPM5 have also been identified as NMT substrates (Guttery et al. 2014). A third PPM protein, PPM6, is also predicted to be myristoylated but has not been experimentally identified (Wright et al. 2014). The expression profile of PPM2 and PPM5 indicates their presence throughout the life cycle, with elevated levels during schizogony and in activated gametocytes. Guttery *et al.* provided evidence for their essential function during sexual and sporogonic development of *P. berghei* through gene disruption experiments. PPM2 was found to be essential for gametocyte sex allocation and ookinete differentiation, while PPM5 modulates oocyst development (Guttery et al. 2014). PPM2 has been found to be essential in the recent *P. falciparum* piggyBac insertion mutagenesis screen while PPM5 is dispensable (Zhang et al. 2018). Both are also suggested to be regulated by phosphorylation (Treeck et al. 2011).

Two likely NMT substrates, calpain (Russo, Oksman & Goldberg 2009) and AK2 (Rahlfs et al. 2009) were not detected by Wright *et al.* in schizonts, probably due to their early expression in asexual blood stages (Wright et al. 2014). Calpain has been localized to the nucleolus (Russo, Oksman & Goldberg 2009), and its successful knock down in *P. falciparum* showed it to be essential for cell cycle progression, with ring stage parasites unable to progress to S-phase (Russo, Oksman, Vaupel, et al. 2009). A non-myristoylated glycine 2 to alanine mutant (G2A) Calpain-YFP chimera expressed from an episomal plasmid accumulated in the cell cytoplasm, indicating the importance of *N*-myristoylation in targeting calpain to the nucleolus (Russo, Oksman & Goldberg 2009). AK2 is expressed primarily in late stage gametocytes

and ookinetes, and largely absent in asexual blood stage parasites. It was found to be essential in the recent *P. falciparum* piggyBac insertion mutagenesis screen (Zhang et al. 2018). Although not identified by Wright *et al.* it was shown to be myristoylated in an artificial co-expression system of recombinant AK2 with NMT (Rahlf s et al. 2009). It was found to be localized beyond the PPM to the PVM by co-localisation with Exported Protein 1 (Exp1) by immunofluorescence imaging (Ma et al. 2012) and to the TVN through PfAK2-RFP co-localisation with Rab5b (Ebine et al. 2016). Combining live fluorescence imaging, a PfAK2[G2A] mutant and biochemical approaches Thavayogarajah and co-workers also found AK2 to be located at the periphery of the parasite attached to the outer leaflet of the PPM and secreted, most likely within the PV beyond the PPM (Thavayogarajah et al. 2015). Transport of AK2 to the TVN is dependent on its myristoylation and palmitoylation (Thavayogarajah et al. 2015). AK2 is therefore an interesting NMT substrate because its location suggests an alternative secretory pathway in *Plasmodium spp.* in which N-terminal myristoylation and adjacent palmitoylation are involved in translocating proteins across the PPM (Thavayogarajah et al. 2015).

1.6 Antimalarial control

Various measures have helped in the fight against malaria, targeting both the mosquito vector and the parasite itself. Three key inventions are at the forefront of the fight against malaria: insecticide treated bed nets (ITNs), indoor residual insecticide spraying (IRS) and artemisinin combination therapies (ACTs) as frontline drug treatments (Bhatt et al. 2015). After positive progress between 2010–2016, with an 18% decline in malaria cases globally (World Health Organization 2017), there has been little progress between 2015-2017 showing that the global malarial response has levelled off (World Health Organization 2018b). Although the goal of the Global Technical Strategy for Malaria 2016-2030 — to eliminate malaria by 2020 in at least 10 countries that had the disease in 2015 — is within reach at the current rate of progress, other countries have reported an increase in cases (World Health Organization 2018b). To get the global malaria response back on track, a new country-driven approach — “high burden to high impact” — was launched in Mozambique in November 2018. This was along with the most recent World Malaria report to help the high burden countries which account for most malaria cases (World

Health Organization 2018b). The approach is anchored by four key elements: improvement on political commitments into resources and tangible actions, improvement on more timely and granular data, improvement on policies and strategies, and a coordinated country response (World Health Organization 2018a).

The most efficacious and cost-effective way to tackle malaria would be through a fully effective vaccine, which has not been developed despite 50 years of research. A vaccine has the advantage of preventing the occurrence of the disease in the first place and therefore preventing transmission. The issue with developing a vaccine against malaria lies within the antigenic diversity of the parasite as well as it being a highly immune-evasive organism. Furthermore, the very complex life cycle (see section 1.3) with various antigenically distinct stages as well as a very short extracellular window where antibodies would be most effective, make a vaccine development very difficult. The efforts over the last 50 years to develop a vaccine can be categorised into three approaches: transmission-blocking vaccines, blood stage vaccines facilitating parasite clearance and clinically silent pre-erythrocytic vaccines. An ideal vaccine requires features spanning all three approaches. This would include raising an immune response against multiple stages including sporozoites, hepatocytes, sexual gametocytes and the asexual blood stages. It should also overcome antigenic variation and genetic diversity by targeting multiple epitopes and thirdly it should be multi-immunogenic by inducing both the humoral and cell-mediated immune response (Arama & Troye-Blomberg 2014). In all three categories vaccine candidates have been generated which fulfil some of those criteria, recently reviewed in Beeson et al., (Beeson et al. 2019). The most success has been made in the past decades with the development of a subunit vaccine especially with RTS,S/AS01. In contrast to whole-organism immunisation strategies, subunit vaccines contain parasite antigens that can also affect the necessary protection. It contains a repeat region of the sporozoite CSP protein fused to Hepatitis B virus surface antigen using a liposomal adjuvant system (Casares et al. 2010). With over 32 years of development process and US\$700 million production cost it is the first licensed vaccine against any kind of parasitic disease (Maxmen 2019). However RTS,S only showed a 27% efficacy in the phase III trial in babies of six to twelve weeks of age while production was higher in young children 5-17 months with 51% after a three dose vaccination (The RTS 2014). The protection drops to 18%

after the four-year follow up showing very short-lived efficacy. This is far from the WHO's target of 75% efficacy (World Health Organization 2015). Nevertheless, the pilot program aims to reach 360,000 children in the three countries and could save thousands of lives. It will be rolled out in phases to identify any further safety issues and determine the feasibility of wider distribution (Maxmen 2019).

1.6.1 Vector control

Vector control strategies aim to prevent transmission of the parasite to the human host through the mosquito vector. This strategy includes reduction of the mosquito population and the prevention of humans being bitten by the mosquito. For the latter, use of ITNs and IRS reduced malarial cases immensely. In 2017, 50% of the population in Africa was protected through ITNs, with an increase from 2010-2015 but no further improvement since 2016 (World Health Organization 2018b). One successful insecticide, mainly used in the 1940s and 1950s and re-permitted in 2011 by the WHO for IRS (World Health Organization 2011), is dichlorodiphenyl-trichloroethane (DDT). Initial success was reduced due to development of resistance by the mosquito to DDT (Brown 1958) and concern about its wider environmental effects (van den Berg 2009; Eskenazi et al. 2009). Four main alternatives have been used since: pyrethroids, organochlorines, carbamates and organophosphates. However, the WHO has reported resistance occurring to all of them (World Health Organization 2016). To reduce the mosquito population, measures such as destruction of mosquito breeding sites resulted in a decrease of cases but changes in climate, increased urbanisation, and changes in land use resulted in an increase in available breeding sites and transmission rates (Caminade et al. 2014). Another strategy to reduce the mosquito population is by the sterile insect technique (SIT) which releases X-ray sterilized males that are unable to produce offspring (Gentile et al. 2015). SIT has been implemented successfully for other vector borne diseases such as the New World screwworm fly — *Cochliomyia hominivorax* — in the USA, but more work is needed to use it for malaria control in a continent-wide setting (Munhenga et al. 2011). Limitations for SIT include the large-scale generation of genetically modified mosquitoes. Improvements in this approach were made by reducing the mosquito's ability to transmit the parasite by genetically engineering the expression of a peptide called salivary gland and midgut peptide 1 (SM1) which

would interfere with ookinete invasion of the midgut epithelium (Ghosh et al. 2001). Any alteration in this way would need to have fitness benefits for the mosquito in order to spread through the population by natural selection. An additional concern with the success of reducing the mosquito population comes from recent findings that *P. falciparum* parasites have evolved a developmental strategy to respond to changes in mosquito reproduction. When the female mosquito reproductive investment is reduced, oocysts develop into sporozoites faster or the number of oocysts per mosquito increases (Werling et al. 2019). This should be taken into consideration for all mosquito population reduction strategies.

1.6.2 Antimalarial chemotherapy

There are four main classes of antimalarial compounds which can be classified by their structure and/or function. These four classes include (1) artemisinin and its derivatives, (2) the quinine derived compounds targeting the blood stages (this includes the 4-aminoquinolines: pyronaridine, piperaquine, amodiaquine and chloroquine, and the aryl-amino alcohols: quinine, lumefantrine and mefloquine), (3) the antifolates (sulfadoxine, pyrimethamine and cycloguanil) and (4) the liver-stage targeting 8-aminoquinolines such as primaquine. Artemisinin combination therapy (ACT) is the first line of treatment for uncomplicated malaria (see section 1.6.2.3). The most important antimalarials are discussed in the following section. All erythrocyte stage-specific antimalarials (excluding the liver-stage specific aminoquinolines) are described with their mechanism of action (MoA) and occurrence of resistance (see section 1.6.3) in Figure 1.6. The figure is taken and adapted from a recent review in 2017 (Blasco et al. 2017).

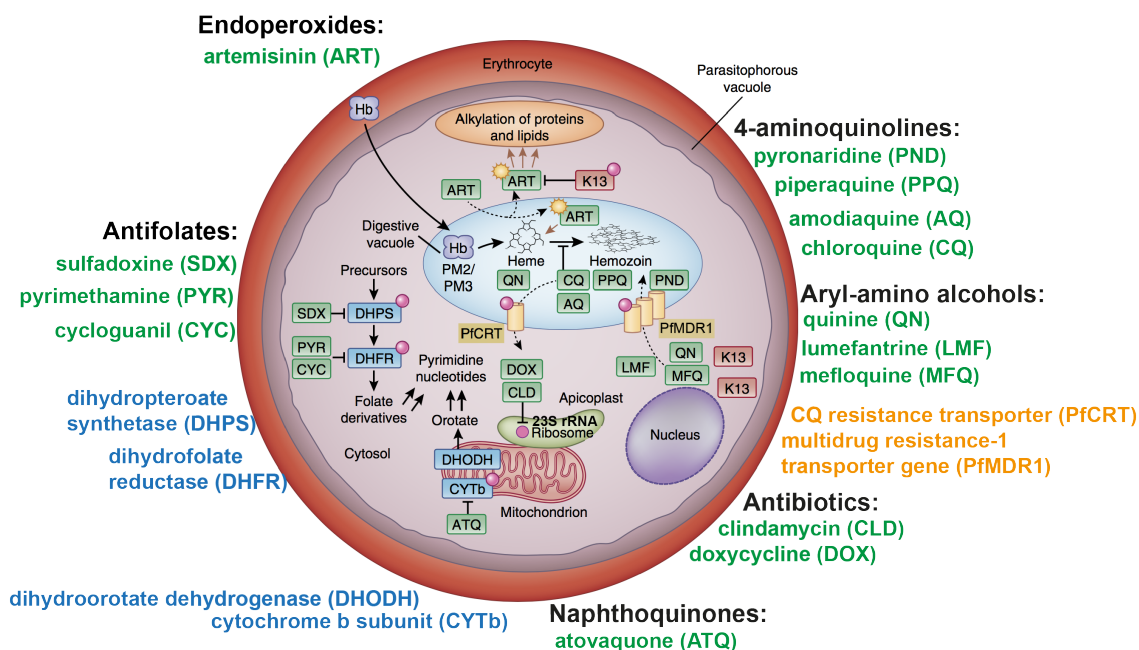


Figure 1.6 Drug resistance mechanisms and molecular targets of antimalarial drugs in the erythrocyte stages.

Figure taken and adapted from Blasco *et al.*, 2017. Frequently targeted biological pathways include folate and pyrimidine biosynthesis in the cytosol, haem detoxification in the digestive vacuole, and electron transport in the mitochondrion. **Drug targets** are shown in blue, **drugs** in green and **transporters** in orange. Abbreviations are explained in the figure.

- **The endoperoxide ART:** haem-mediated cleavage of an endoperoxide bridge in the ART structure (star symbol) leading to the generation of carbon-centred free radicals causing intracellular damage via selective alkylation.
 - Resistance: **point mutation** (pink circle) in **K13**, that localizes at the endoplasmic reticulum (ER) and in vesicular structures
- **The 4-aminoquinolines:** bind reactive haem in the digestive food vacuole, and interfere with its detoxification by incorporation into chemically inert hemozoin
 - Resistance: **point mutation** (pink circle) in genes encoding transporter **PfCRT** and **Pgh1**, increases expression of aspartic proteases plasmepsin 2 and 3 (PM2/PM3) in the digestive vacuole.
- **The Aryl-amino alcohols:** disruption of haemoglobin digestion during the blood stage of the parasite
 - Resistance: Copy-number changes in **pfmdr1** (encoding transporter **Pgh1**), as well as **PfCRT** and **pfmdr1** sequence variants
- **Antifolates:** targeting the folate biosynthesis pathway
 - Resistance: **point mutation** (pink circle) in two key enzymes of the folate biosynthesis pathway, **DHPS** and **DHFR**
- **Naphthoquinones:** **ATQ** inhibits mitochondrial electron transport
 - Resistance: single point mutation (pink circle) in the **CYTb** of the bc1 complex
- **Antibiotics:** **CLD** and **DOX** inhibit protein translation inside the apicoplast
 - Resistance: single **point mutation** (pink circle) in the apicoplast-encoded **23S rRNA**

1.6.2.1 Quinine and derivatives

One of the most effective treatments of malaria started in 1820 with the first isolation of quinine from the bark of the *Cinchona* tree (Achan et al. 2011). Since 2006, quinine is no longer used as a front-line drug with resistance first occurring in the 1980s (Bunnag et al. 1996). Since resistance to quinine is a rare occurrence (Achan et al. 2011), it is still used for severe cases if no other treatments are available (K. D. Miller et al. 1989). A quinine derivative is chloroquine, developed in 1934 to reduce the side effects and increase the cost-effectiveness of quinine (Loeb et al. 1946). Resistance occurred only 15 years after its first deployment in the 1950s (Eyles et al. 1963) and its usage is now only for *P. vivax* treatment in regions where resistance has not developed (see section 1.6.3) (Tse et al. 2019). To tackle resistance a second quinine derivative, which is still used today, called mefloquine was developed in the 1970s (Trenholme et al. 1975). Resistance was first reported shortly after in 1986 (Brasseur et al. 1986), and it has been shown to have central nervous system toxicity affecting a large number of patients (Nevin & Croft 2016). Quinine derivatives are commonly used in combination with a complementary drug such as mefloquine and artesunate, to reduce the chance of resistance. Other quinine derivatives developed include: amodiaquine synthesized in 1948 (Berliner et al. 1948) and still used for uncomplicated *P. falciparum* infections and piperazine, developed in 1960 and structurally related to chloroquine (L. Chen et al. 1982), which is still used today as a partner drug with dihydroartemisinin. The MoA of quinolone-related drugs is thought to be through the disruption of haemoglobin digestion during the blood stage of the parasite (Foley & Tilley 1997). The toxic haem can no longer be sequestered into the haemozoin crystal in the food vacuole. This is because chloroquine acts as a weak base and binds haem forming a toxic complex (Homewood et al. 1972) as well as preventing haemozoin formation by inhibiting the haem polymerase (Slater & Cerami 1992). Quinine is thought to act by a similar mechanism although the interaction with haem is thought to be weaker (Slater 1993). Mefloquine on the other hand has been shown to act through a different mechanism by acting on the parasite cytoplasmic ribosome and inhibiting protein synthesis (W. Wong et al. 2017).

1.6.2.2 Antifolate antimalarials

Pyrimethamine and sulfadoxine are two antifolate antimalarials developed in the 1950s (pyrimethamine) and 1960s (sulfadoxine) (Tse et al. 2019). Both drugs are no longer used in isolation due to high levels of resistance but a combination was approved in 1981 and is now sold under the trade name Fansidar® (Tse et al. 2019). Both drug are effective against all asexual blood stages as well as early gametocytes by targeting the folate biosynthesis pathway, with pyrimethamine inhibiting dihydrofolate reductase (DHFR) and sulfadoxine inhibiting dihydropteroate synthase (DHPS) (Lumb et al. 2011).

1.6.2.3 Artemisinin combination therapy

Artemisinin is the active compound extracted for the first time in 1979 by Tu Youyou from the sweet wormwood plant *Artemisia annua* (Qinghaosu Antimalaria Coordinating Research Group 1979). The plant has been used against fever in traditional Chinese medicine for centuries. It is now a crucial anti-malarial therapy, especially with resistance to quinoline-containing and antifolate antimalarials. Artemisinin is thought to act by generating free radicals which cause damage to many essential proteins via selective alkylation, disrupting many biological processes of the parasite (Wang et al. 2015). The activation of artemisinin and generation of the highly reactive carbon-centred free radicals involves the haem-mediated cleavage of an endoperoxide bridge in the structure of artemisinin (Meshnick 2002; Klonis et al. 2013). The success of artemisinin was limited by its bioavailability, which led to the generation of semi-synthetic derivatives including the three most common: artemether, artesunate, and arteether (Paddon & Keasling 2014). However, the short half-life as well as concerns over development of resistance led to the ACT strategy, which combines an artemisinin derivative (fast-acting, short half-life) with a partner drug with a longer half-life (long-acting) and a different MoA. Due to the rapid killing by artemisinin derivatives and the longer half-life of the second drug, the probability of resistance development was minimized and made ACTs the global gold standard of first-line treatment for uncomplicated malaria. However, even though it took a long time to emerge, in 2008 the first cases of artemisinin resistance were reported (Noedl et al. 2009) (see section 1.6.3). Furthermore, as valuable as the ACTs are, they fail to kill mature gametocytes and

therefore do not block transmission (Tilley et al. 2016). Neither do they kill dormant liver-stage hypnozoites of *P. vivax* infections (Douglas et al. 2010).

1.6.2.4 Liver-stage targeting 8-aminoquinolines

Currently the only drugs available to target hypnozoites, a dormant form of the parasite persisting in the liver and causing multiple relapses of non-*falciparum* species including *P. vivax*, are primaquine and tafenoquine. The MoA of both drugs is not well understood and both cause dangerous side effects in patients who are glucose-6-phosphate dehydrogenase (G6PD)-deficient as they can induce the severe blood disorder acute haemolytic anaemia (Alving et al. 1956). G6PD-deficiency is an X-linked enzymopathy found in malaria-endemic areas, making it a serious issue for treatment. Tafenoquine was first discovered in 1978 and recently approved as the first single dose drug against *P. vivax* in over 60 years (Ashley & Phyo 2018). It has an improved efficacy compared to primaquine (Q. Li et al. 2014).

1.6.3 Development of resistance against antimalarials

Selective pressures that drive drug resistance can differ throughout the life cycle of the parasite. Factors that drive drug resistance can include: parasite number, stage-specific drug action, the essentiality of the drug target, and host immunity. There are now at least nine genes that are known to be involved in drug resistance development in *P. falciparum* (Figure 1.6). These include resistance mechanisms through point mutations as well as copy number polymorphism (CNP). Point mutations have been identified in genes encoding two key enzymes of the folate biosynthesis pathway: **DHFR** (pyrimethamine resistance) (Peterson et al. 1988) and **DHPS** (sulfadoxine resistance) (Triglia et al. 1997), in the gene encoding the chloroquine resistance transporter (**PfCRT**) (Chloroquine/quinine resistance) (Fidock et al. 2000) and **Pgh1** (quinine, chloroquine, mefloquine, lumefantrine, artemisinin resistance) (Cowman et al. 1994), in the gene encoding the propeller domain of the **K13 kelch protein** (artemisinin resistance) (Ariey et al. 2014) as well as in the **cytochrome b** subunit (atovaquone resistance) (Vaidya 2011). CNP have been identified in GTP cyclohydrolase I (**GCH1**) (pyrimethamine resistance) (Heinberg et al. 2013), as well as of the haemoglobin-digesting proteases **plasmepsin 2 and 3**

(piperaquine resistance), and multidrug resistance-1 transporter (*pfmdr1*) (chloroquine resistance) (Cowman et al. 1994).

Artemisinin resistance was first reported in 2008 in Cambodia (Noedl et al. 2009), with a study reporting the time for parasite clearance increased from 48h to 84h (Dondorp et al. 2009). From 2008-2019, 30 independent cases of resistance in southeast Asia were reported, with the largest number of cases to dihydroartemisinin-piperaquine combination associated with a mutation in the propeller domain of the K13 kelch protein (Ariey et al. 2014). The slower clearance time means that chances of resistance occurring to the partner drug will also rise. K13 is predicted to be a substrate adaptor of an E3 ligase (Gupta & Beggs 2014). The point mutation in the K13 kelch protein is thought to reduce affinity for a particular protein substrate, reducing ubiquitination and proteasomal degradation, thereby decreasing the artemisinin induced protein damage, proteopathy and death of the parasite (Mbengue et al. 2015). This substrate is the phosphatidylinositol-3-kinase (PfPI3K). The prevention of its ubiquitination and degradation results in elevation of its lipid product phosphatidylinositol-3-phosphate (PI3P) (Mbengue et al. 2015). This causes expansion of PI3P-vesicles from the ER which may neutralize the artemisinin-induced toxic proteopathy through the induction of proteostasis pathways including biogenesis, correct protein folding, trafficking, vesicular remodelling and degradation, and the ubiquitin-proteasome system (UPR) pathway (Bhattacharjee et al. 2018).

Chloroquine resistance occurred only 15 years after its development (Eyles et al. 1963) and quickly spread over 40 countries (Payne 1987). The resistance comes from multiple mutations in the parasite encoded transporter expressed in the digestive vacuole membrane encoded by the *Pfcr1* gene (Fidock et al. 2000). The mutations such as Lys76Thr enable efflux of chloroquine from the digestive food vacuole, thereby reducing the toxic effect of the drug. Another transporter protein, also localized in the digestive food vacuole, is P-glycoprotein homolog 1 (Pgh1) and encoded by the *Pfmdr1* gene (Cowman et al. 1991). Pgh1 is the basis of another resistance mechanism against chloroquine through amplification and therefore overexpression of its gene. Mefloquine resistance develops through mutations in the coding region of Pgh1 (either through CNP or point mutations) (Cowman et al. 1994).

Non-synonymous point mutations in DHFR and DHPS can lead to the development of resistance to antifolate antimalarials pyrimethamine (Peterson et al. 1988) and sulfadoxine (Triglia et al. 1997). In addition, as for chloroquine, CNP can also result in resistance to the pyrimethamine-sulfadoxine combination therapy. In this case the first enzyme in folate biosynthesis, GTP-cyclohydrolase 1, is amplified leading to resistance (Nair et al. 2008).

Interestingly, resistance to most antimalarials has appeared in the same geographical area: the Greater Mekong Subregion, especially Cambodia. This includes resistance to quinine and chloroquine (Eyles et al. 1963), as well as resistance to artemisinin (Noedl et al. 2009) and the ACTs such as dihydroartemisinin-piperaquine (Leang et al. 2013). Multiple factors might have favoured the emergence of multi-drug resistance in Cambodia. For example, factors revolving around host immunity, which is typically lower in Asian settings as humans are less exposed to the malaria parasite compared to in high-transmission countries in Africa (Blasco et al. 2017). There is a weaker ability to clear the drug-resistant infection naturally, due to the lower level of antimalarial immunity in Asia. Disorders such as G6PD deficiency and haemoglobinopathies are also highly prevalent in Southeast Asian population and might play a role as they alter the redox state of RBCs. Therefore, they might influence the occurrence of ART resistance which act in part by increasing oxidative stress (Blasco et al. 2017).

Overall the emergence of resistance has highlighted the growing urgency for development of antimalarial drugs with novel MoAs. To maintain the progress made in antimalarial drug discovery there is a need to discover targets and pathways which are not compromised by existing drug resistance. It is also important to think about drug resistance early on in the drug discovery process to find ways to circumvent it.

1.6.4 The future of antimalarial chemotherapy

The potential of new compounds to act as agents for antimalarial chemotherapy is judged by numerous requirements established by the Medicine for Malaria Venture since 1999. This includes: a single-dose cure, as opposed to current drugs such as

artesunate and chloroquine which need to be given in multiple dosages, a novel MoA without any cross-resistance to any of the current drugs, activity against both gametocytes responsible for transmission and the sexual stages both together preventing infection, as well as the ability to target *P. vivax* hypnozoites (Tse et al. 2019). There is a great interest in drugs that act on gametocytes and hypnozoites in order to stop transmission, with some drugs now in pre-clinical trials (e.g. SC83288 developed in 2017 and active against early stage gametocytes (Pegoraro et al. 2017)).

There are several approaches to discovery new anti-malarial drugs. One is the exploration of new formulations and combinations of current anti-malarial drugs to overcome issues of resistance and make them more effective. This involves, for example, overcoming K13-mediated ART resistance through the synthesis of a more stable peroxide bond compared to other artemisinin derivatives with a view to yielding a longer half-life (e.g. the compound Ozonide OZ439 (Charman et al. 2011; Phyo et al. 2016)). A different approach is to circumvent the induction of UPR and 'proteostasis' pathways associated with the stress response of artemisinin resistance by inhibiting the *P. falciparum* 20S proteasome (H. Li et al. 2016). The proteasome inhibitor WLW-vs showed good efficacy in a rodent *in vivo* model of malaria, and a cryoelectron microscopy structure was used to guide further compound design (H. Li et al. 2016).

Alternatively, existing drugs developed for other diseases can be repurposed for malaria, bringing the advantage of already knowing the MoAs. The compounds often already have a good pharmacokinetic profile, are already approved by the U.S. Food and Drug Administration (FDA) or the European Agency for the Evaluation of Medicinal products (EMA) as safe or have undergone phase II clinical trials, and therefore serve as a good starting point for antimalarial drug discovery. One example of combining both strategies is Imatinib, a cancer therapy drug which has been repurposed and combined with dihydroartemisinin-piperaquine in a triple combination now in phase II trials (Tse et al. 2019).

There are two broad strategies that have steadily controlled the process of early stage drug discovery over the last hundred years: the empirical phenotypic drug

development (PDD) relying on the compound-induced phenotype in a physiological relevant *in vivo* or *in vitro* system such as in cell, tissue or whole organism; and the molecular or theoretical target-based drug development (TDD) approach that tends to be predominantly hypothesis driven focusing on a target gene product to modulate its activity in an *in vitro* assay (Swinney 2013). The major key difference between those two approaches is the significance of target identification. While TDD requires the unambiguous identification of a molecular target, PDD is based on an approach without the need of the prior identification of a target. Historically in the classical forward genetic approach during a PDD process, the phenotype of interest is studied under experimental selection pressure through a compound with an unknown MoA and unknown target(s) (Swinney 2013). Many antimalarials now in pre-clinical trials have been discovered through the phenotypic route starting off with large libraries of compounds in an *in vitro* assay. This included compounds such as M5717 (DDD107498) originating from a phenotypic screen in Dundee with protein kinase scaffold compounds against multi-drug resistant *P. falciparum* (Baragaña et al. 2015). M5717 shows equal potency against drug-resistant strains, is active against multiple life cycle stages, and is as effective as other antimalarial drugs in the *in vivo* *P. berghei* mouse model (Baragaña et al. 2015). The target of M5717 was identified to be translation elongation factor 2 (eEF2) and the drug showed complete blood-stage parasite clearance as well as showing the ability to act as a transmission blocking drug against mature gametocytes (stage IV-V) (Baragaña et al. 2015). M5717 is now in Phase I clinical trials with healthy volunteers in Australia (Tse et al. 2019). Another example originating from a phenotypic screen and showing a potential in clinical trials is UCT943 that has shown single dose curing ability *in vivo* (Le Manach et al. 2016). Over the past two decades many compounds have been screened against the malaria parasite leading to new starting points for malaria drug discovery (Gamo et al. 2010; Guiguemde et al. 2010) including the generation of an open access MMV “Malaria Box” of confirmed drug-like compounds with blood stage activity (Spangenberg et al. 2013). Although high-throughput phenotypic screening will continue to play an important role in antimalarial drug discovery, the target based rational drug design also produced some lead candidates now in phase II trials. Examples are drug targets involved in the electron transport chain (ETC) targeted by atovaquone: cytochrome bc₁, NDH2 (Biagini et al. 2012) and dihydroorotate dehydrogenase (DHODH) (Coteron et al. 2011). Another example is DHFR, the

target of antifolate antimalarials (pyrimethamine) (Yuthavong et al. 2012). In three cases, resistance development to previous drugs led to an enzyme activity-based high throughput screen to identify the selective inhibitors (cytochrome bc1, NDH2 and DHODH). In all four cases a structure-guided approach using co-crystal structures with inhibitors along with efficacy and pharmacokinetic profiling led to the design and characterisation of new potent inhibitors. In the case of DHFR, the structure-guided approach identified the inhibitor P218 which binds both wild-type and pyrimethamine resistant PfDHFR mutant (Yuthavong et al. 2012).

1.7 *N*-myristoyltransferase as a drug target in *Plasmodium*

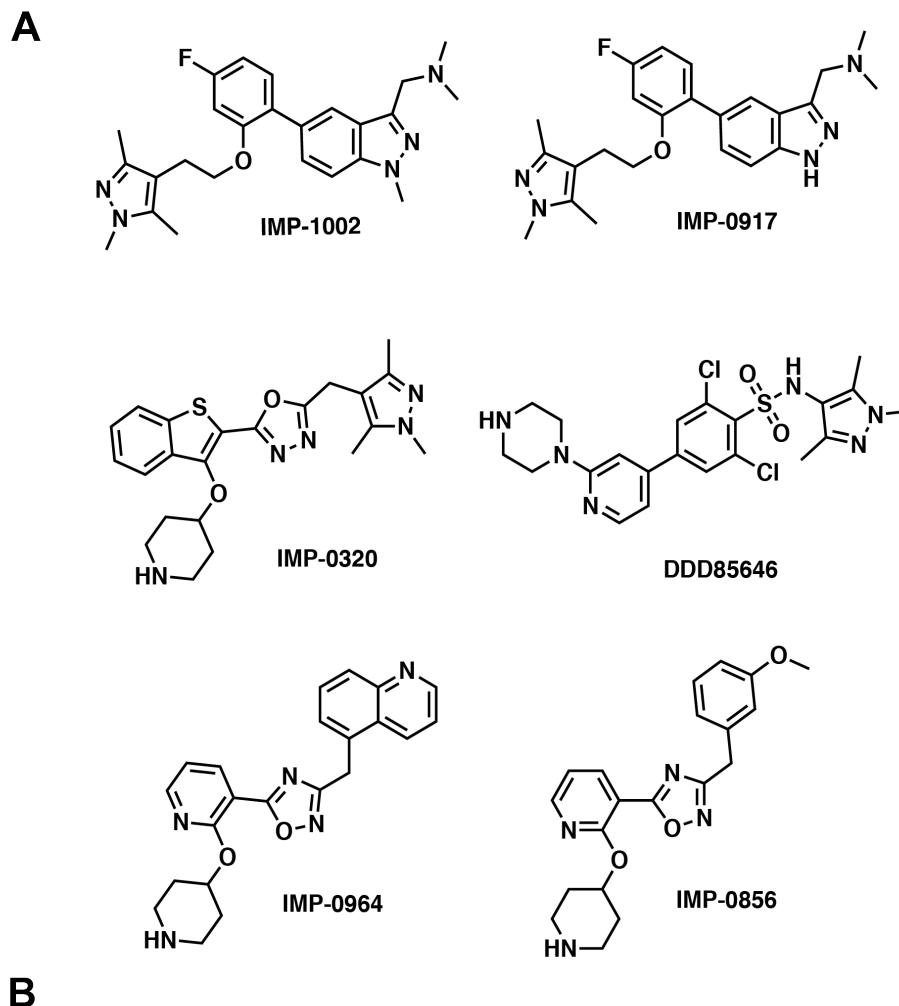
Another example of a target-based rational drug design is against the enzyme *N*-myristoyltransferase (NMT) (see section 1.4). Myristoylation is an essential modification for *Plasmodium* with many substrates involved in crucial biological processes (see section 1.5). In order to develop species-specific inhibitors targeting NMT, the less conserved peptide binding pocket (see section 1.4.2) has been the focus of the drug discovery process. Selectivity over the human host's NMT (HsNMT1 and HsNMT2) is particularly important when designing a suitable antimalarial inhibitor. The knockdown of NMT activity using pharmacological NMT inhibitors (NMTi) has been used successfully to investigate the phenotypic consequences of NMT inhibition without the need to generate a conditional genetic knockout of the essential and constitutively expressed single *nmt* gene in *P. falciparum* (Wright et al. 2014) (see section 1.7.2). In a tetracycline-induced knock-down of the *nmt* gene in *P. berghei* (Pino et al. 2012), parasites showed a severely impaired maturation resulting in abnormal morphology of erythrocyte schizonts, reminiscent of what was observed by Wright *et al.* following NMTi treatment of *P. falciparum* parasites (Wright et al. 2014).

1.7.1 NMT inhibition by small molecules

NMT was first considered as a drug target against the human pathogen *C. albicans* (Weinberg et al. 1995) and has since been considered as a drug target for many infectious diseases (see section 1.4). An early *P. falciparum* NMTi example is the compound DDD85646 (compound 1a PDB: 2YND), first reported as an inhibitor

against *Trypanosoma brucei* NMT (Frearson et al. 2010) but has been used in *Plasmodium* *in vitro* and *in vivo* with moderate potency, and induced toxicity *in vivo* (Wright et al. 2014). To improve potency for PfNMT a target-based drug discovery project involved two larger high-throughput screening (HTS) approaches over the years. The first HTS was performed using 60,000 compounds from MRC technology (MRCT), screened through a fluorescent-based assay against PvNMT (Goncalves, Brannigan, Whalley, et al. 2012). The assay exploits the reaction between the thiol-selective fluorescent dye CPM (7-diethylamino-3-(4-maleimido-phenyl)-4-methylcoumarin) and the free CoA which is liberated during the enzymatic acyl-transfer reaction (Goncalves, Brannigan, Thion, et al. 2012) (see section 2.2.3.4). From the MRCT HTS the most promising hit was developed into a series based on a quinoline scaffold (Goncalves, Brannigan, Whalley, et al. 2012; Goncalves et al. 2017). Additionally, a “piggy-back” approach using the CaNMT inhibitors in a small library screen with 25 compounds, led to the development of a benzo[b]furan scaffold series against *Plasmodium* (Yu et al. 2012). Although some of the compounds reduced parasitaemia *in vitro*, the major challenges of those early series were low ligand efficiency (LE), due to their high molecular weight and high lipophilicity combined with low enzyme affinity as well as poor cellular uptake (Rackham et al. 2014). Improvements were made with a series based on a benzo[b]thiophene scaffold that mediated improved stacking-interactions with two tyrosine residues (Tyr211 and Tyr334) close to the substrate binding pocket with the increased aromatic character of the thiophene moiety compared to the benzo[b]furan scaffold (Yu et al. 2012; Rackham et al. 2013). One compound from this benzo[b]thiophene series (compound 2a, IMP-0320 PDB: 2YNE) (Figure 1.2) was tested in an *in vivo* *P. berghei* mouse model by Wright et al. IMP-0320 (2a) significantly reduced parasitaemia over the course of three days (50 mg/kg intraperitoneally, twice a day) and did not induce acute toxicity compared to DDD85646 (1a) (Wright et al. 2014) (Figure 1.7). The addition of a methoxyphenyl substituent, in the benzo[b]thiophene series, increased the π - π interaction even further to other amino acids (Ser319, Phe105) (Rackham et al. 2014) but the compounds of this series still had a high lipophilic LE (LLE) value resulting in the inhibitor being able to bind multiple targets (promiscuity) and therefore toxicity. The benzo[b]thiophene scaffold was further developed into a pyridyl inhibitor by replacing the bicyclic core (Yu et al. 2012). This

series included IMP-0964 and IMP-0856 (PDB ID: 4UFX) (Yu et al. 2012) (Figure 1.7) which both show *in vitro* activity but selectivity over human NMT remained low.



NMTi name	Publication	DOI of paper	PDB # WT	PDB # G386E
IMP-1002	Schlott et al., 2019	10.1016/j.chembiol.2019.03.015	6MB1	6MB0
IMP-0917	Mousnier et al., 2018	10.1038/s41557-018-0039-2	5O6H	—
DDD8646	Wright et al. 2014	10.1038/nchem.1830	2YND	6MAZ
IMP-0320	Wright et al. 2014	10.1038/nchem.1830	2YNE	—
IMP-0964	Yu et al., 2015	10.1039/c5md00242g	—	—
IMP-0856	Yu et al., 2015	10.1039/c5md00242g	4UFX	—

Figure 1.7 NMT inhibitors used in this thesis.

A. Chemical structures of NMT inhibitors. B. Table of NMT inhibitors with their publication and PDB # if a crystal structure is available of NMT with bound inhibitor. PDB # G386E refers to resistance mutant discussed in chapter 5 only, but is mentioned here for completeness.

A second large HTS was performed to address the limitation with the previous series. This screen was run against 150,000 compounds from Pfizer in a radioactivity-based scintillation proximity assay (SPA) against both PfNMT as well as *Leishmania donovani* NMT (Bell et al. 2012). Two main series were identified which contained compounds that showed selectivity for PfNMT over HsNMT. One of them is the aminomethylindazole series including compounds IMP-0917 (PDB: 5O6H) (Mousnier et al. 2018), and IMP-1002 (PDB: 6MB1) (Schlott et al. 2019) (Figure 1.7). This HTS and the following medicinal chemistry investigation also resulted in several potent human NMTi (e.g. IMP-1088) which are leads with antiviral activity *in vitro* (Mousnier et al. 2018). The most significant interaction of the aminomethylindazole series with NMT is between the pendant phenyl ring and two tyrosine residues (Tyr211 and Tyr334) forming π - π stacking interaction (Tyr211) and edge-face interactions (Tyr334). For the stacking interaction, Tyr211 has to adopt an alternative conformation which has previously been hypothesized to result in selectivity over HsNMT1 (Yu et al. 2012). The conformation change observed in PvNMT in Tyr211 when a *Plasmodium* selective NMT inhibitor is binding allows the stacking interaction to occur. The HsNMT1 equivalent residue, Tyr296, is hypothesized to have a higher energy barrier associated with this alternative conformation, resulting in steric hindrance of the *Plasmodium* selective inhibitor with Tyr296 (Yu et al. 2012). The flexibility of Tyr211 to adopt different conformations is also the basis of the resistance mechanism against IMP-1002 discussed in Chapter 5.

The lack of success for NMTi to reach clinical trials as antimalarial compounds is based on several factors. This includes that most NMT orthologues in species of the same genus such as *Plasmodium* are structurally distinct resulting in a narrow spectrum of possible activity to target NMT in different *Plasmodium* species (e.g. *P. vivax*). On the other hand, selectivity over other NMT of species such as humans can be difficult to achieve due to high homology. Another challenge is that a high level of enzymatic activity is necessary to generate the necessary cellular activity. This resulted in work to improve potency aiming to increase cellular activity. Additionally, this increase in potency is often restricted to molecules with a basic centre that mimic the N-terminus of the substrate peptide and interacts with the C-terminal carboxylate of the enzyme which can result in some undesirable physicochemical properties limiting cellular uptake in some cases.

1.7.2 The NMT inhibition phenotype

Selective NMT inhibition for the entire erythrocyte life cycle leads to severe pleiotropic consequences affecting parasite development, presumably due to the malfunction of multiple substrates and the resulting downstream effects leading to an irreversible failure of parasites to complete their life cycle (Wright et al. 2014). When myristoylation is inhibited from an early ring stage (NMTi 4x EC₅₀ inhibition 1 - 48 hours post invasion (h PI)) development is blocked at around 36 - 39 h PI, arresting the parasite at a stage with 4 - 7 nuclei compared to control parasites with ~20 nuclei at 48 h PI (Wright et al. 2014). NMTi-treated parasites fail to develop to fully mature schizonts leading to a significant drop in parasitaemia in the subsequent cycle. It has been hypothesized that these abnormal “pseudoschizonts” with 4 - 7 nuclei are arrested in development due to a failure to assemble the IMC during early schizogony, which is in turn due to the loss of essential IMC components, including NMT substrates such as GAP45, ISP1, and ISP3 (Wright et al. 2014). The expression of IMC markers (e.g. GAP45, MyoA, MTIP and ISP3) is greatly reduced in NMTi treated parasites, suggesting that parasite development arrests before 38 h PI, a stage when GAP45 synthesis is normally complete (Ridzuan et al. 2012; Wright et al. 2014). It is also possible that the defect precedes the onset of the “pseudoschizont” phenotype and might be initiated by an earlier malfunction. Since treatment with a NMTi for 48 h generates a severe phenotype, it is important to investigate the consequence of changing the duration and timing of the treatment window. Such experiments may help in determining a shorter lethal inhibition time and help understand the molecular basis of NMT inhibition.

1.8 Chemical biology tools to study myristoylation in *Plasmodium*

In silico analysis of the *P. falciparum* genome for potential NMT substrates indicated that up to 2% of the 5400 encoded proteins are NMT substrates (Wright 2013). Those prediction tools are helpful but are not programmed to work with databases containing *Plasmodium* proteins. Without a dataset from a closely related organism the predictors are not necessarily able to generate the data. Nevertheless, they indicate the low abundance of myristoylation substrates in the proteome, which

results in a need to enrich the modification in order to study it. Experimentally, just over 30 substrates have been identified in the schizont stage of parasite development in erythrocytes through a chemical proteomic approach using a myristic acid analogue (YnMyr) to label and enrich NMT substrates (Wright et al. 2014) (Figure 1.8).

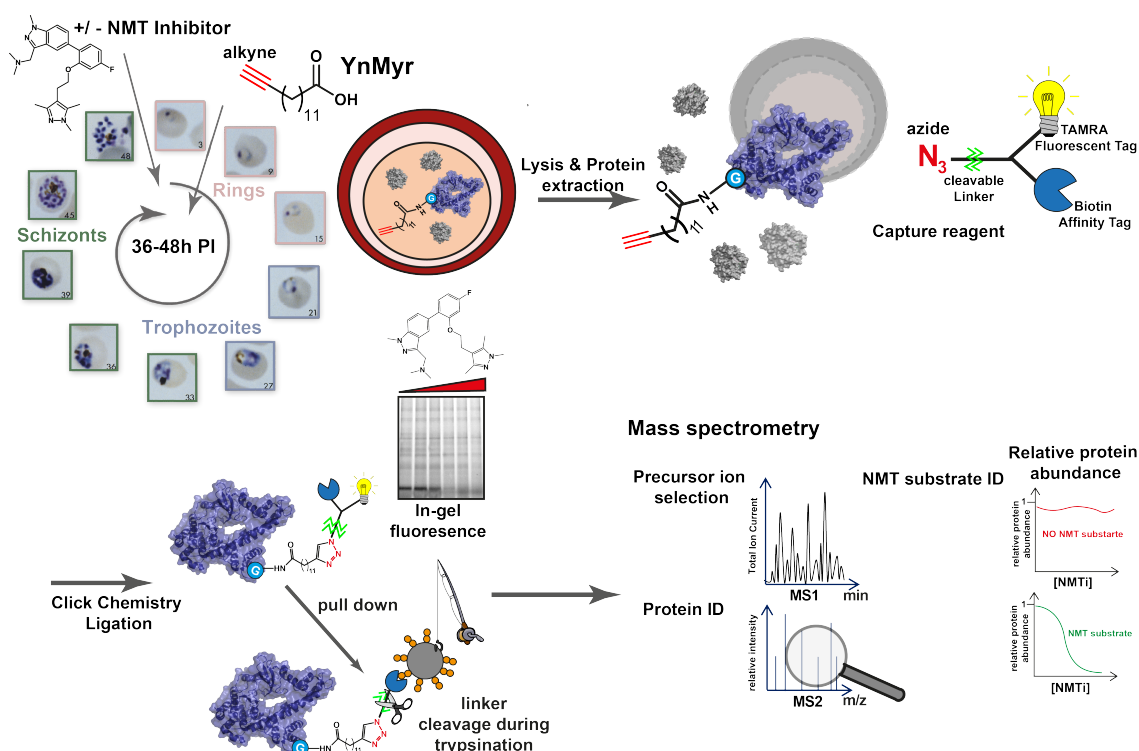


Figure 1.8 Comparative chemical biology workflow.

Synchronized parasite cultures are incubated with YnMyr, in the presence of NMT inhibitor (NMTi) or DMSO carrier during the developmental stage of interest. Red blood cells (RBCs) containing parasites are then lysed and proteins are extracted. The alkyne handle on YnMyr is ligated to a multifunctional capture reagent containing biotin and/or TAMRA fluorophore through Cu(I)-catalyzed azide/alkyne cycloaddition (CuAAC, 'click' chemistry). The fluorophore is used for in-gel visualization of NMT substrates. The biotin allows NMT substrate enrichment on streptavidin coated beads. An additional optional cleavable linker allows the fluorophore and biotin handle to be removed after pull down and enables direct identification of the site of modification by MS/MS. After on-bead digestion with trypsin, NMT substrate-derived peptide fragments are expected to decrease in samples with increasing NMTi concentration. Peptide fragments resulting from fragmentation of the reporter ions are submitted to MS2, generating individual spectra for each peptide. Figure taken from Schlott et al., 2018.

This metabolic tagging with click chemistry (MTCC) is a complementary approach that is generally applicable to a variety of lipid modifications including myristoylation

(YnMyr) and palmitoylation (YnPal). It relies on the principle of using a chemical analogue of the lipid PTM containing a biorthogonal tag to be attached onto the substrate by the endogenous enzyme. In the case of YnMyr, myristic acid containing an alkyne group as the bioorthogonal chemical tag is attached biosynthetically to the N-terminal glycine through NMT. The alkyne tag is small and generated by changing a single to a triple bond containing the same number of carbon atoms. YnMyr is highly mimetic of myristic acid and well tolerated by NMT (Wright et al. 2014). The alkyne handle, in turn, can be used to attach a capture reagent containing an azide group through copper-catalysed azide/alkyne cycloaddition (CuAAC) (Lang & Chin 2014) (Figure 1.8).

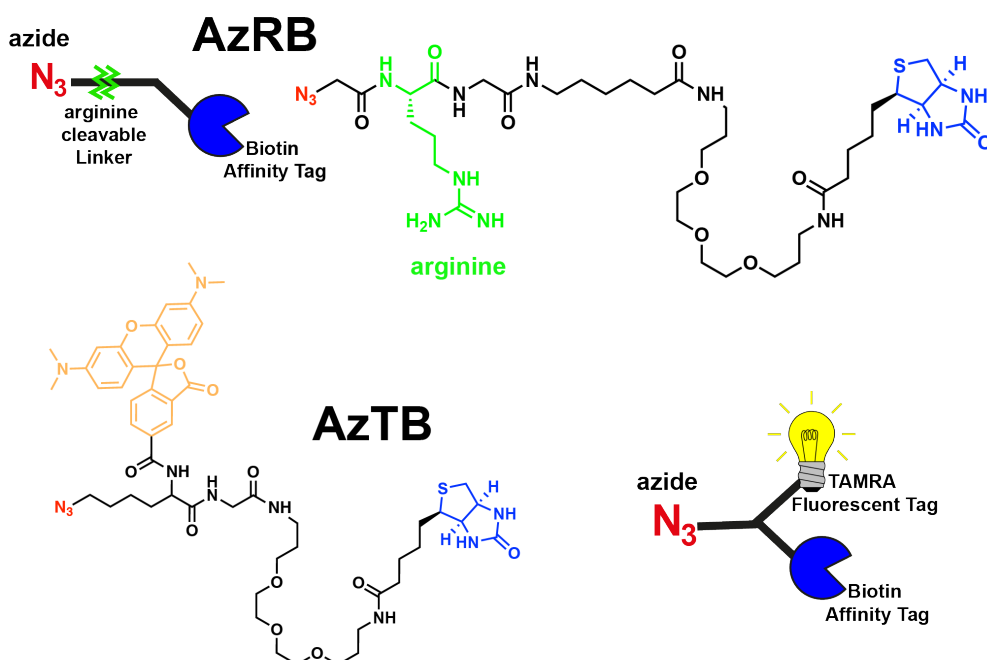


Figure 1.9 Structures of capture reagents

Shown are AzRB used for proteomics and AzTB used for streptavidin pull down and in-gel fluorescence. Tetramethylrhodamine (TAMRA) in yellow, azide capture group in red, biotin in blue and cleavable arginine linker in green.

The capture reagent may contain a fluorophore (TAMRA) (AzT) for visualisation by in-gel fluorescence, a biotin moiety (AzB) for subsequent purification using streptavidin coated beads followed by identification by Western blotting or mass spectrometry (MS), or both (AzTB) (Figure 1.9). Having the alkyne group attached to the lipid rather than the capture reagent has been shown to lead to reduced

background labelling since the alkyne group is much less reactive (Wright et al. 2014). Being able to detect the N-terminal peptide attached to the capture reagent, is appealing because it allows for the identification of the site of modification through MS. However, this can be quite difficult because of the large molecular weight and the hydrophobicity of the biotin-lipid-peptide fragment and the difficulties to separate the high affinity biotin-streptavidin interaction. This obstacle has been addressed by using a cleavable biotin-azide capture reagent that contains an arginine that is recognised by trypsin to allow selective release of the labelled peptide (called AzRB with biotin and AzRTB with biotin and TAMRA) (Figure 1.9) (Broncel et al. 2015). The peptide with the site of modification is cleaved between the alkyne and the biotin group resulting in a free, smaller fragment to be analysed by MS (Figure 1.8). To study the response of myristoylation inhibition *in vitro*, quantitative and comparative chemical proteomics use a potent and selective NMTi as a tool to discriminate NMT substrates from non NMT-substrates. As outlined in Figure 1.8, YnMyr is used in both NMTi treated and control samples to enrich for NMT substrates. The enrichment is selectively inhibited through the presence of the NMTi in the treated sample, leading to a reduction of NMT substrate myristoylated, despite the same abundance of proteins being synthesized. The reduction in myristoylation through NMT inhibition can be visualised both through in-gel fluorescence or MS.

Another possible way to use bioorthogonal functionalities is to study S-acylation through acyl biotin exchange (ABE) (see section 2.2.4.7). This method exploits the ability to selectively cleave thioesters to attach an affinity tag replacing S-acylation. Treatment with a thiol-reactive reagent such as N-ethylmaleimide (NEM) blocks free thiols in the protein lysate of interest. This allows for the selective cleavage of all thioester S-acetylation through hydroxylamine and the subsequent addition of either a HPDP-biotin label for pull down (Roth et al. 2006) or a polyethylene glycol (PEG) tag to introduce a mass shift visualized through Western blotting (Percher et al. 2016).

1.8.1 The proteomic LC-MS/MS System

The method of choice when performing proteome-wide discovery experiments such as the identification of myristoylation, is proteomics using mass spectrometry (MS)-based proteomics. MS enables a highly accurate and sensitive detection of the mass of an analyte by converting it into a gaseous, charged form. Through the application of electrostatic potentials, the ratio of the mass of each ion to its charge can be measured (Cañas et al. 2006). A range of instruments are available and technological advances have been reviewed recently (Yates 2019). During MS/MS the mass analysis of a sample is followed by the selection of a specific ion with a specific mass-to-charge ratio (m/z). This precursor ion is then fragmented and subjected to a second round of mass analysis (van Agthoven et al. 2019). In top-down proteomics, proteins are directly analysed by MS/MS and therefore the whole protein is analysed (Q. Wu et al. 2012). In bottom-up proteomics on the other hand proteins are fragmented into peptides via chemical or enzymatic digestion and the continuing workflow uses the digested peptide fragments (Q. Wu et al. 2012). Digested peptides are subjected to high pressure liquid chromatography (HPLC), and then drawn into the aerosol phase by electrospray ionisation (ESI). The peptide ions are then detected according to their ion intensity and retention time, and directed to a mass analyser such as an Orbitrap detector with high resolving power. In a second round of MS, ions can be isolated and sent to a fragmentation chamber where peptides are broken down by high-energy collision dissociation (HCD). This results in a mixture of fragment ions which can be resolved in the Orbitrap with an MS2 spectrum to obtain the sequence of the peptide, which is searched against a database containing genome-derived protein sequences (Cantin & Yates 2004).

Throughout this thesis, either a Q Exactive or an Orbitrap Fusion Lumos Tribrid mass spectrometer was used. The advantage of the Lumos is an increased scan speed leading to an increased number of peptide (hence protein) identifications through the five different ion separation/storage devices (Yates 2019). Furthermore, it has advantages for the quantification of samples labelled with isobaric tags as it allows the production of an MS3 spectrum through the Tribrid mass spectrometer function (see section 1.8.2).

1.8.2 Quantification Methods in Proteomics

Two different quantification methods were used in this thesis. One being label-free quantification (LFQ) and the other being quantification through peptide labelling with an isobaric (same mass) Tandem Mass Tag™ (TMT). For LFQ the signal intensity of the peptide precursor ion from a particular protein between two samples was measured and compared (Bantscheff et al. 2007). An alternative form of LFQ is to count and compare the number of fragment spectra (spectral counts) identifying peptides of a given protein (Bantscheff et al. 2007). For LFQ, each sample is individually run and intensities compared afterwards during the data analysis. TMT labelling offers the advantage of multiplexing up to eleven samples (each sample is labelled with a different isobaric tag), which reduces experimental variability from the point of sample mixing on as well as reduce machine running time, which is generally quite costly. TMT reagents label primary amines (N-terminal and on the lysine side chains) of peptides with an isobaric tag that is only broken into two (a reporter ion of a specific mass for each tag, and a spacer that counteracts the mass difference of the reporter ion) at the MS2 level. Because the tags are chemically almost identical and have the exact same mass, it results in co-elution at the MS1 mass spectrum due to the same retention time. The difference in mass and therefore quantification is revealed by the different fragmentation of the isobaric tag in the MS2 into its reporter ions, which are proportional to the intensity of a peptide in each given sample (MS2-level quantification) (Thompson et al. 2003). Both the precision and the accuracy of the quantification can be affected if contaminating peptide ions with a similar m/z are isolated together with the target peptide ion leading to ratio distortion and an interference effect (Ting et al. 2011). An additional purification through a third MS round generating an MS3 spectrum can separate the target peptide from any co-eluting contaminant peptides which is possible with the Orbitrap Fusion Lumos Tribrid mass spectrometer (Ting et al. 2011). But this additional purification step, improving the accuracy and precision of quantification, comes at a cost of decreased acquisition speed and sensitivity (Wühr et al. 2012). Therefore, there is still some debate whether an MS3-level quantification is beneficial or contains too many drawbacks. Alternatively, research has gone into improving the accurate quantification of TMT-labelled peptides at the MS2 level without the addition of ion

purification (Wühr et al. 2012) or by improving the sensitivity of the MS3 by increasing the number of reporter ions in the MS3 spectrum (McAlister et al. 2014).

1.9 Molecular biology tools to study the role of myristoylation in *Plasmodium*

1.9.1 Genetic manipulation using CRISPR-Cas9 and repair through homologous recombination

Genetic manipulation through reverse genetic studies in *P. falciparum* go back to 1997 with the first targeted knockout of the knob-associated histidine-rich protein (KAHRP) (Crabb et al. 1997) showing that KAHRP is essential for the formation of knobs on the RBC surface. Traditional methods of integrating exogenous DNA into the *P. falciparum* genome by homologous recombination (HR) relied on the stochastic generation of a double strand break (DSB) in the genome close to the gene of interest, in order for HR to occur with the provided repair plasmid. HR is the only repair mechanism in *Plasmodium*, unlike the situation in most other eukaryotes which perform canonical non-homologous end joining (NHEJ) (Aravind et al. 2003). For the haploid life cycle stages (see section 1.3), not repairing the DSB would be lethal and therefore the repair is necessary. Conventionally, a single homology region was used for a single crossover integration which inserted a selectable drug cassette (e.g. WR99210 (WR) (Fidock & Wellems 1997)) by repairing the DSB. This insertion duplicates part of the targeting sequence rather than replacing it, which can lead to reversion to wild type sequence by an additional HR event between the duplicated regions and the loss of the integrated region. HR by double crossover with two homology regions either side of the integrated region facilitates replacement of the region between the homology arms. The region to be integrated is recodonised to allow HR to only occur adjacent to the target region, replacing the endogenous sequence. Due to the stochastic generation of the DSB, the time frame for generating positive integration was several months and very inefficient. Furthermore, with this conventional method, the drug selection marker is integrated into the genome which limits the potential for serial modification as not many drug resistance markers are available.

A technique which can induce targeted DSB is called the clustered regulated interspaced protospacer repeat (CRISPR) – CRISPR-associated protein 9 nuclease (Cas9) system (CRISPR-Cas9). The CRISPR-Cas9 system originates from *Streptococcus pyogenes* where it functions as an adaptive immune mechanism of the bacteria against invasion by foreign DNA and viruses (Seruggia & Montoliu 2014). Cas9 is an endonuclease which uses single guide RNA (sgRNA) to guide itself to a target sequence where it inserts a DSB into the genome. The sgRNA comprises 20 bp that need to match the target sequence followed by a 3 bp protospacer adjacent motif (PAM) (NGG or NAG) which functions as the Cas9 DNA binding domain. The system was adapted for *P. falciparum* in 2014 (Ghorbal et al. 2014) using a proof of concept experiment to introduce an artemisinin-resistance mutation into the genome of wild type parasites.

1.9.2 Genetic manipulation using the Cre recombinase conditional system

The genome of *Plasmodium* erythrocyte stages is haploid (see section 1.3) resulting in no establishment in culture if an essential single copy gene is deleted or mutated to generate a lethal phenotype. This does not allow any phenotypic characterization of parasites lacking the protein of interest or of the lethal mutation. The Cre recombinase system can be used to conditionally excise a stretch of DNA (Figure 1.10). Using a topoisomerase I-like mechanism, the Cre recombinase from bacteriophage P1 mediates excision of DNA between two 34 bp sequences called *loxP* (Sternberg & Hamilton 1981). *LoxP* consists of two 13 bp inverted repeats, separated by an 8 bp nonpalindromic spacer region (Hoess et al. 1986). The name for Cre recombinase originates from its function to **Cause RE**combination while the *loxP* is located at the **locus of X**(crossing)-over in the bacteriophage **P1** (Sternberg & Hamilton 1981). The rapamycin-induced dimerization system (DiCre) allows the activation of the Cre recombinase only upon addition of rapamycin (Jullien et al. 2003; Jullien et al. 2007). The major helical Cre recombinase binding domains are separated into two fragments each fused with a different rapamycin binding domain (FRB and FKBP12) which will only dimerize upon the addition of rapamycin, activating the Cre recombinase (Figure 1.10). The system was adapted for the use in *P. falciparum* in 2013 by excising the *SERA5* gene using this inducible system.

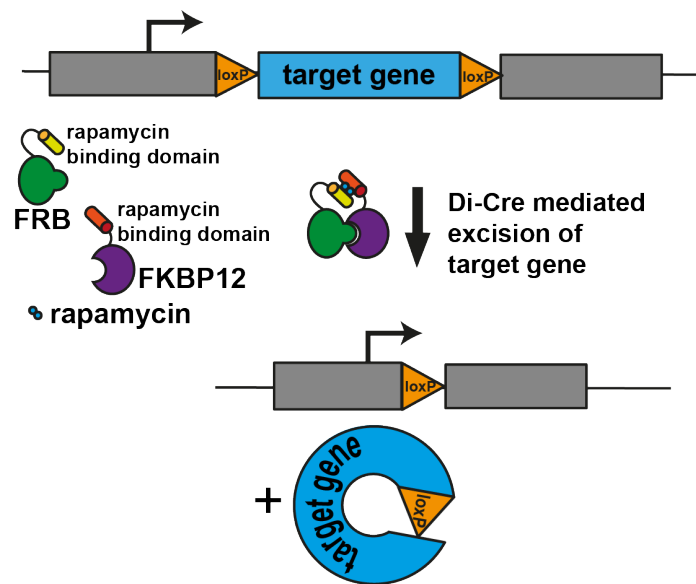


Figure 1.10 Cre recombinase dimerization.

Activation of the Cre recombinase through dimerization upon addition of rapamycin leads to excision of the target gene flanked by two loxP sites. The two Cre recombinase fragments FRB and FKBP12 contain two rapamycin binding domains, dimerizing and therefore activating the recombinase which will excise DNA sequence between two loxP sites leaving the excised product and the excised locus each with one loxP site.

The study produced a clone which constitutively expresses the two dimerisable fragments of the Cre recombinase called 1G5DC (Collins, Das, et al. 2013). Issues with this parasite line came from the loss of the DiCre cassette due to genetic rearrangement since the line was generated through a single cross-over recombination (Sherling et al. 2017). To address this the clone was modified using the CRISPR-Cas9 system to remove 1 kb of the duplicated 5' end of the *SERA5* gene in order to prevent spontaneous excision of the DiCre components (clone was named B11) (Perrin et al. 2018). Two additional parasite lines were generated in the Holder lab by Ellen Knuepfer, where the DiCre recombinase was inserted into two different blood stage dispensable loci: *p230p* and *pfs47* (Knuepfer et al. 2017). This line has since been used with success and has not shown any instability.

1.10 Project aims and objectives

Malaria is one of the most significant infectious diseases worldwide, and the escalating threat of parasite resistance to all current antimalarial drugs highlights the need for new drugs with novel modes of action. The aim of this thesis is to:

- further study *N*-myristoyltransferase (NMT) as a novel antimalarial target. This includes exploring protein *N*-myristoylation in the malaria parasite by using chemical proteomics to identify NMT substrates that show a lethal phenotype when their myristoylation is inhibited in isolation.
- study the essentiality of myristoylation through generic approaches including a conditional N-terminal glycine to alanine substitution for NMT substrates as well as complementation of an NMT substrate knock out with a G2A variant.
- provide genetic evidence for the on-target activity of several classes of NMT inhibitors through resistance selection. The aim is to use a structure-guided approach to identify and overcome resistance to NMT inhibitors.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Chemical tools for click chemistry and NMT inhibitors

The following chemical tools were synthesized as described previously: YnMyr, AzTB, AzRB2 (Heal et al. 2008; Heal et al. 2011; Broncel et al. 2015), DDD85646 (Frearson et al. 2010), IMP-0964, and IMP-0856 (Yu et al. 2012), IMP-0320 (Wright et al. 2014), IMP-0917 (Mousnier et al. 2018), IMP-1002 (Schlott et al. 2019). All other chemicals were purchased from either Sigma-Aldrich, VWR or NovaBiochem and used without further purification.

2.1.2 Molecular biology reagents

Oligonucleotide primers (Table 2.1) were synthesized by Sigma-Aldrich. Rapamycin (R0395) was also obtained from Sigma-Aldrich and was prepared in dimethyl sulfoxide (DMSO) to 200 μ M. All restriction enzymes were purchased from New England Biolabs. The antifolate drug WR99210 (WR) from Jacobus Pharmaceuticals was stored in DMSO, as a 20 μ M stock.

Primer #	Primer name	Chapter	Sequence
1	Gap45_SUTR_Spel_F1.1	Chapter 4	ATATATACTAGTTTATCATGCCTTTTAAACAATAATATATATATTATACAC
2	Gap45_SUTR_EcoRI_HpaI_R1	Chapter 4	ATATATGTTAAACATATATGAATTCATTATATAATATTGAATACAGTAAAAAATTTAAAG
3	Gap45_V1_EcoRI_LoxP_Nterm_F3	Chapter 4	ATATATGAATTCATAACTTCGTATAGCATACATTATACGAAGTTATATGGGTAACAAGTGCAGTAGGAGTAAGGTTAAAGAGCCAAA GAGAAAGGATATTGATGAACCTTGACAGCGTGAAAACTCTC
4	Gap45_V1_LoxP_CNterm_GA_R3	Chapter 4	GAGCACTTGTTGCCATATAAAGTCGTATAATGTATGCTATACGAAGTTATTCAGCTCAGGAGCGGTGTGTCGG
5	Gap45_V2_LoxP_Nterm_GA_F4	Chapter 4	CGAAGTTATATGGCAAAACAAGTGCTCTCGTTCAAGGTGAAAGAGCCGAAGAGGATATCGACGAAGTGGCGGAACGCTG
6	Gap45_V2_AS_PhosMu_R4	Chapter 4	GGTAACACTACGATAGGTGAAATAGATTGACTCCTC
7	Gap45_V2_AS_PhosMu_F5	Chapter 4	GAGGAGTCAATCTATTTACCATCTCGTAGTGTTACC
8	Gap45_V2_Cterm_NotI_HpaI_R5	Chapter 4	ATATATGTTAAACATATATGCGGCCGCTTACGATAACAGAGGGGTATCACTCAGATCGATTTTG
9	Gap45_3UTR_NotI_F2	Chapter 4	ATATATGCGGCCGCATTAAAGTTAAACATTAACAAAAACAAAAAATTTAAATAAAG
10	Gap45_3UTR_HpaI_R2	Chapter 4	ATATATGTTAACGAACCAATATAAAAAATCCTTATTAAATATAATAAAC
11	Gap45_SUTR_Spel_F1	Chapter 4	ATATATACTAGTGAATTTGTTTACATACACTGTGTAATCC
12	Integ_G45_gWT_R	Chapter 4	GGTACTTGTTACAGGTCTCTCTCTTC
13	Integ_G45_gWT_F	Chapter 4	GAGGAAGAAGAACCTGAACAAGTACC
14	Integ_G45_3UTR_R	Chapter 4	gatttcgatgaattttaatttttttaatac
15	S_Gap45_R1	Chapter 4	CAATTTTCTGTCGTCCAATTCCT
16	S_Gap45_F3	Chapter 4	GAAATCGATTACGCGACTCAGGAA
17	S_Gap45_SUTR_F4	Chapter 4	aaacgtatggaagtgtaaaggg
18	Gap45_A2G_Rew - NcoI	Chapter 4	ATATATCCATGGCATAGTCCGGGACGTCATAGGGATAG
19	S_Gap45_R3	Chapter 4	CCTGAGTCGCGTAATCGATTTCTT
20	S_Gap45_F1	Chapter 4	CAAGAATTGGACGAACAAAAATT
21	pL6MCSfor	Chapter 4	CTATATACTAGGAATACTAAATATATATCCAATG
22	pL6MCSrev	Chapter 4	gctgcccagacttttagactagaataagc
23	Intg_ARO_SUTR_F2	Chapter 4	gaacaaataagtggtcaaatagattacgc
24	Intg_ARO_WT_SUTR_R2	Chapter 4	TATACCAAACTCTTGATGTTTATTTTATAAAGTAAactg
25	Intg_ARO_WT_SUTR_F2	Chapter 4	aggatatttgtagctctttttcatgagtc
26	Intg_ARO_3UTR_R2	Chapter 4	aagttgtttccacttgattcacc
27	ARO_Intg_G2A_R1	Chapter 4	TATAGTCTTTGGATCTGCTGCC
28	ARO_Intg_G2A_R2	Chapter 4	CAAGAACTCTGGAATTTCAACACC
29	ARO_Mfel_STOP_F	Chapter 4	atatatCAATTGGAAGCTATATACCATTTAGAAGATTTT
30	AJP_161	Chapter 4	gtgtatatttactctacattatctcc
31	AJP_162	Chapter 4	tatgactgttactctgtagtgac
32	AJP_169	Chapter 4	gcccaactgtatgttggtcatc
33	AJP_164	Chapter 4	cattcctaacacattatgtgtataaca
34	AS_GAP45C_Intg_WT3UTR	Chapter 4	gtcaggggattaaataaaatac
35	AJP_163	Chapter 4	ATTGAGCAGAGGATATGCGCATAATGGT
36	G45_WTC_pbDT3_R	Chapter 4	GCACACAACATACACATTTTACAG
37	Integ_G45_3UTR_R	Chapter 4	gatttcgatgaattttaatttttttaatac
38	AJP_93	Chapter 4	tgtttaatacatactgtgtatccctt
39	GAP45_AJP37_SnaBI_F	Chapter 4	ATATATTACGATTGATAAATTAATACATATATATATATATATAAAAAATTAACGtatgg
40	GAP45_AJP37_AflII_R	Chapter 4	CGAGTAACATAACGTAACCGCTCTTAAG ATATAT
41	GAP45_AJP37_G2A_F	Chapter 4	CAATATTATATAATAATGGCAATAAATGTTCAAG
42	GAP45_AJP37_G2A_R	Chapter 4	CTTGAACATTTAATTCGTCATTATATATAATATTG
43	ARO_Xmal_STOP_R	Chapter 4	atatatGGGCCCTTAATCGTCAACGC
44	ARO_Intg_F1	Chapter 3	TATTATACCGTTGCTTTCAAAATGGCGGG
45	ARO_Intg_R1	Chapter 3	CATACATCTCTACCGGCGCAACAG
46	GAP45_Intg_F1	Chapter 3	aaacgtatggaagtgtaaaggg
47	GAP45_Intg_R1	Chapter 3	CTCGTCTATGTCCTTTCTCTTTGGC
48	ISP3_Intg_F1	Chapter 3	GGAAAAAGTTTTATGTTTCAGTCTGAATATTACC
49	ISP3_Intg_R1	Chapter 3	CTTTAATTGAGTTACCTGATTTGTACTTGTTAATCC
50	S9C_Intg_F1	Chapter 3	gaaataaatacatgttaacacatagttataagataacc
51	S9C_Intg_R1	Chapter 3	TTACTGTAACCTGGAGGATGTGGCC
52	TRP_Intg_F1	Chapter 3	GAAAAATCATTCTCTTAATTCATATTG
53	TRP_Intg_R2	Chapter 3	CCACTTCTAATAACTTCATACTTGCG
54	CDPK1_Intg_F1	Chapter 3	cattttgatggtgcaactgctttttgagg
55	CDPK1_Intg_R1	Chapter 3	ACCGTAGTTGTTACCGTTTGTGAACCTTGACC
56	CDPK1_WT_R1	Chapter 3	CCATTCTGAATTTACTTCTCTCGC
57	ARO_WT_R1	Chapter 3	ATCTCTCTCGCACAGCAATTATTTC
58	GAP45_WT_R1	Chapter 3	TTTCATCAATATCTTACGTTTGGGTTC
59	ISP3_WT_R1	Chapter 3	CTGAAATATATCTATATTGATTACTG
60	S9C_WT_R1	Chapter 3	CTTTGAATAGCTAGTGGGTGCGGCTGT
61	TRP_WT_R1	Chapter 3	TTTCTGCTAGCATAATGTAATACTCC
62	ARO_MiSeq_F1	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTATTATACCGTTGCCCTTCAATGGC
63	ARO_MiSeq_R1	Chapter 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATACATCTCTACCGGCGCAACAG
64	GAP45_MiSeq_F1	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGtaaacgtatggaagtgtaaaggg
65	GAP45_MiSeq_R1.2	Chapter 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCGTCTATGTCCTTTCTCTTTGGC
66	ISP3_MiSeq_F1	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAAAAGTTTTATGTTTCAGTCTGAATATTACC
67	ISP3_MiSeq_R1	Chapter 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTTAATTGAGTTACCTGATTTGACTTGTTAATCC
68	S9C_MiSeq_F1	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGgaaataaatacatgttaacacatagttataagataacc
69	S9C_MiSeq_R1	Chapter 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTACTGTAACCTTGAGGATGTGGCC
70	TRP_MiSeq_F1	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAAAACATTTCTCTTAATTCATATTG
71	TRP_MiSeq_R2	Chapter 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCACTTCTAATAACTTCATACTTGCG
72	CDPK1_MiSeq_F1	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGgaaaaataaatacgtagaataatgtttcccc
73	CDPK1_MiSeq_R1	Chapter 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGTGTGTTACCGTTTGTGAACCTTTGACC
74	GAP45_MiSeq_F1.2	Chapter 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGtaaacgtatggaagtgtaaaggg
75	G386E Sequencing F	Chapter 5	TATGGCAAGCTATATACAGCAGG
76	G386E Sequencing R	Chapter 5	CCTCAGACTATATAACATTAGAATG

Table 2.1 Oligonucleotide primers used in this thesis.

In each figure where a primer is illustrated the number will refer back to the Oligo # in this table. Red residues in MiSeq primer indicate complementary region with target sequence, while black sequence indicates the overhang adapter sequence.

Name	Species	Working dilution		Working dilution IFA	Source/reference if applicable
		Western			
anti-PfGAP45	Rabbit	1:13000	polyclonal affinity purified	1:2500	Rees-Channer et al. 2006
anti-PfGAP45	Mouse		polyclonal serum	1:50	unpublished Roxanne R Rees-Channer Thesis
anti-PfCDPK1	Rabbit		polyclonal affinity purified		Green et al. 2008
anti-PfBip	Rat	1:10.000	polyclonal affinity purified		Knuepfer et al., 2013
anti-PfMTIP	Rabbit	1:3000	polyclonal affinity purified		Green et al. 2006
anti-PfARO	Rabbit	1:2000	polyclonal affinity purified		Anthony Holder, The Francis Crick Institute (Kaviri)
anti-PfARF	Rabbit	1:1000	polyclonal serum	1:500	Anthony Holder, The Francis Crick Institute (Ruani)
anti-PvNMT	Rabbit a	1:750	polyclonal serum		PvNMT protein made by Jim Brannigan at York University and Antibodies raised by David Moss
anti-HSP70	Rat	1:1000	polyclonal serum		Ellen Knuepfer, The Francis Crick Institute
anti-CDPK4 calmodulin domain	Rabbit	1:2000	polyclonal serum		Anthony Holder, The Francis Crick Institute (Babs)
anti-MyoA	Rat (Rat 2)	1:500	polyclonal serum		Judith Green, The Francis Crick Institute
anti-GAP50	Rabbit	1:1000	polyclonal affinity purified 1.53 mg/ml	1:500	Judith Green, The Francis Crick Institute
anti-SERA5	Rabbit	1:1000	polyclonal serum (test bleed)	1:500	Collins et al., 2017
anti-MSP7	Rabbit	1:2000	polyclonal serum		Knuepfer
anti-PfMSP1 5.2	Mouse	1:3000	polyclonal affinity purified 5.6mg/ml	1:1000	Ellen Knuepfer, The Francis Crick Institute
anti-PfEBA175	Rat		polyclonal affinity purified	1:500	Ellen Knuepfer, The Francis Crick Institute
anti-PfRON4 mAB 24CF3	Mouse		polyclonal affinity purified	1:500	Jean-Francois Dubremetz, University Montpellier France
anti-HA 3F10	Rat		monoclonal affinity purified	1:500	Roche
anti-Rat - biotin conjugate	Goat		polyclonal affinity purified	1:1000	Novax
anti-His	Mouse	1:500	polyclonal affinity purified		Santa Cruz SC8036 Lot B2317

Table 2.2. Primary antibodies used in this thesis.

Working concentrations is given for Western Blot and IFA separately. =

Name	Type	Animal	Target	Working concentration	Source
Streptavidine-Alexa 594	polyclonal	n.a	biotin	1:1000	Invitrogen
Anti-Mouse/Rabbit/Rat HRP	Polyclonal	Goat	Mouse/Rabbit/Rat FC chain	1:2500	Sigma & BioRad
Anti-Mouse/Rabbit/Rat	Polyclonal	Goat	Mouse/Rabbit/Rat FC chain	1:5000	Invitrogen
Alexa Fluor 488					
Anti-Mouse/Rabbit/Rat	Polyclonal	Goat	Mouse/Rabbit/Rat FC chain	1:5000	Invitrogen
Alexa Fluor 594					

Table 2.3 Secondary antibodies used in this thesis. Conjugated to Alexa Fluor dyes or horseradish peroxidase (HRP)

2.1.3 Antibodies

Polyclonal antibodies were generously gifted by several people outlined in Table 2.2 with their corresponding publication if available. The remaining antibodies were purchases (source shown in table). Secondary antibodies used in this thesis can be found in Table 2.3.

2.2 Methods

2.2.1 *P. falciparum* cell culture.

2.2.1.1 *In vitro* maintenance and synchronisation of *P. falciparum* parasites

All *P. falciparum* work described in this thesis used the 3D7-WEHI strain originating from the Walter and Eliza Hall Institute. The 3D7-WEHI-p230pDiCre (p230p-3D7) was acquired from Ellen Knuepfer and recently published in (Knuepfer et al. 2017). The *gap45:ha3:loxP* conditional GAP45 knock-out line was generated in B11 (DiCre expressing line) parasites (see section 1.9.2) and acquired from Abigail Perrin (Perrin et al. 2018). Asexual RBC stage *P. falciparum* parasites were cultured *in vitro* in RPMI-1640 medium containing 0.5 % w/v Albumax II (ThermoFisher Scientific), 0.0292 % w/v L-glutamine, 0.005 % w/v hypoxanthine, 0.23 % w/v sodium bicarbonate, 0.0025 % w/v gentamicin sulphate, 0.5957 % w/v HEPES and 0.4 % w/v dextrose to give a compete medium (cRPMI). Parasites were kept at 2-4 % haematocrit. Blood was acquired from anonymous donors, provided through the UK Blood and Transfusion Service. Parasite cultures were gassed with 90 % N₂, 5 %

CO₂ and 5 % O₂ and incubated at 37°C, as published in (Trager & Jensen 1976). Parasite growth was routinely examined by Giemsa stained air-dried thin RBC smears fixed with methanol, followed by visualisation by light microscopy to determine the developmental stage. Parasitaemia was determined by counting a minimum of 500 RBCs.

Parasites were synchronized by isolating mature, schizont-stage parasites using a 70 % Percoll gradient (GE Healthcare Life Sciences) to purify schizont stages with a subsequent reinvasion in a shaking incubator at 37°C at ~100 rpm for 30 min – 2 h depending on synchronicity needed. For very tight synchronization, any remaining intact schizonts were removed by a second Percoll purification. This was followed by a treatment with 5% D- sorbitol to remove the remaining mature parasites from the RBC pellet (Trager & Jensen 1976).

2.2.1.2 Cryopreservation and thawing of *P. falciparum*-infected RBCs

Cryopreservation was carried out by re-suspending infected RBCs (iRBC) containing 3-5% rings in an equal volume of sterile malaria freezing solution (MFS, 111 mM NaCl, 28% glycerol and 3% D-sorbitol). 200-300 µl of packed iRBC were re-suspended in 700 µl of MFS dropwise and transferred to a cryovial (ThermoFisher Scientific), which was stored in liquid nitrogen.

Thawing of cryopreserved cultures required malaria thawing solution (MTS, 3.5% NaCl). Each cryovial was warmed to 37°C before being gently re-suspended in 700 µl sterile MTS. Spinning at 115 x g (1200 rpm in Heraeus Megafuge 16) for five minutes gently pelleted the RBCs, which were then re-suspended in MTS and washed twice more in MTS. The resulting pellet was resuspended in 10 mL cRPMI, and the parasites then cultured as standard.

2.2.1.3 Isolation and extraction of genomic DNA from *P. falciparum*

To allow haemolysis to extract DNA, 0.15% (w/v) saponin (Sigma-Aldrich) in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) was added to pelleted cultures at a minimum parasitaemia of 4%. Centrifugation

at 16060 x g for five minutes was followed by aspiration of the (haemoglobin-rich) supernatant. The resulting pelleted parasite material was stored at -80°C until required. Parasite genomic DNA (gDNA) was extracted from the thawed pellets using a DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions.

2.2.1.4 Transfection of *P. falciparum*

DNA constructs were introduced to mature schizonts of either p230p-3D7 DiCre parasites (for the *gap45*[G2A] and *aro*[G2A] lines) or *gap45:ha3:loxP* parasites (B11 background) for the *gap45:ha3:loxP::comp_gap45*[G2A] as previously described in (Moon et al. 2013; Collins, Das, et al. 2013). For the G2A screen and the PfNMT[G386E] lines, 3D7 parasites were used. Mature schizont-stage parasites were obtained by Percoll-enrichment and a minimum 20 µL pellet of washed and packed schizonts was used for each transfection. The DNA construct(s) to be transfected (see section 2.2.2.5) were mixed with 100 µl P3 Primary cell solution from the P3 Primary cell 4D Nucleofector X Kit L (Lonza). Schizonts were then re-suspended in the DNA/Primary Cell solution mix, transferred to a cuvette and electroporated using the FP158 programme of the Amaxa 4D Electroporator machine (Lonza). Immediately after electroporation, the contents of the cuvettes were transferred to 2 mL of pre-warmed cRPMI at a haematocrit of 15%, and incubated for one hour at 37°C. Parasites were incubated under conditions of gentle shaking (~ 60 rpm) to discourage invasion of the same RBC by multiple parasites. Parasites were incubated, static, at 37°C after diluting iRBC to 3 % HC with 8ml cRPMI. The culture was fed with fresh cRPMI after ~24 h post transfection to facilitate removal of cell debris that accumulates due to electroporation. Selections were carried out as recently described (Knuepfer et al. 2017). Parasites were cultured in the presence of 2.5 nM WR99210 drug pressure for five days allowing selection of parasites transfected with plasmids carrying the human dhfr selectable marker on the pDC2 (Cas9/guide) plasmid. Transfected parasites usually reappeared after 15-17 days. After 25-30 days, following establishment of the transgenic parasites and freezing through cryopreservation (see section 2.2.1.2), gDNA was extracted (see section 2.2.1.3) to enable the detection of the extent of the integrated construct in the mixed population through polymerase chain reaction (PCR) (see section 2.2.2.1).

Positive selection with 1-10 μM 5-fluorocytosine (5-FC) provided as clinical grade Ancotil® (MEDA) was initiated for one week to minimize the existence of excess pDC2 (Cas9/guide) plasmid. Transfected parasites were cloned by limiting dilution (Rosario 1981) at 0.3-0.5 parasites per 100 μl at 0.75 % HC in flat-bottomed 96-well plates and screened for single parasites with a plaque assay. Wells with a single plaque were transferred into a round bottom 96-well plate after around 8-10 days and moved into 48-well plates with 500 μl at 3% HC after another 7-8 days (J. A. Thomas et al. 2016). Alternatively, the 0.3-0.5 parasites per 100 μl at 2 % HC were added to 96-well round bottom plate and clones were detected through colour change of the medium after around 18-20 days and transferred to 48-well plates with 500 μl at 3% HC. After a subsequent 3-4 days incubation at 37°C with regular feeding with fresh cRPMI parasites were smeared and screened by scraping 4 μl blood from the bottom of the well which is diluted into 20 μl of sterile TE and boiled for 3 cycle with each a 3 min boiling phase at 96°C and a subsequent cooling phase for 30 sec at 52°C. The boiled RBC were used to add 1 μl into a 24 μl GoTaq mixture to screen Individual clones by PCR amplification.

2.2.1.5 Selection of IMP-1002-resistant *P. falciparum* Dd2 parasites using a single-step strategy

Prior to selection, genomic DNA was harvested and a frozen stock of the parental Dd2 parasite line was generated. The Dd2 clone used was recloned from the Dd2 parental line by limiting dilution. Selection was initiated with three wells of 10^6 parasite inocula and three wells of 10^7 parasite inocula. A concentration of 120 nM IMP-1002 (3.4 x the 35 nM EC_{50}) was added to a predominantly ring stage culture. The culture was fed daily with drug-containing complete medium for six days and every other day thereafter. The parasitaemia was monitored and kept below 10 % over the next days to prevent overgrowth. Fresh RBCs (equivalent to 0.5 % hematocrit) were added at day 7. At day 14, and weekly thereafter, the cultures were passaged ($\frac{3}{4}$ cultured cells were replaced $\frac{1}{4}$ with fresh RBC and new medium) to prevent lysis. Drug pressure was maintained for 60 days.

2.2.1.6 Conditional excision of *LoxP*-flanked gene through Rapamycin treatment

Immediately after invasion, synchronous ring-stage parasite cultures were divided in two and re-suspended in cRPMI containing either 10 nM rapamycin in DMSO (stock solution 200 μ M in DMSO), 10 μ M stock in cRPMI of which 10 μ L added to 10 ml culture) or an equivalent volume of DMSO to act as the vehicle control. Parasites were treated with DMSO or rapamycin for 1-4 h at 37°C before being pelleted by centrifugation, washed once and returned to culture in cRPMI. The shortest exposure time and rapamycin concentration required to achieve maximal recombination levels of DiCre and still exceed 99% excision levels is 10 nM rapamycin for 30min (Knuepfer et al. 2017).

2.2.1.7 Diagnostic PCR detected integration and excision events

To assess integration of transfection constructs into the *NMT*, *GAP45*, and *ARO* genomic loci, as well as the G2A screen of *GAP45*, *CDPK1*, *ARO*, *S9C*, *TRP* (tetratricopeptide-repeat protein) and *ISP3*, diagnostic PCRs were carried out on extracted gDNA (see section 2.2.1.3). For PCR, the GoTaq Green Master mix from Promega was used as a polymerase as per manufacturer's instructions, with appropriate primers (Table 2.1). PCR products were examined by SYBR Safe (ThermoFisher Scientific) stained agarose gel electrophoresis. Table 2.1 shows the primer combinations used for the various products to determine 5' and 3' integration (Primer #44-61). In each case this involved one primer outside of the region of homology and one within the integrated construct. The wild type (WT) specific primer pair for each integration event is shown as well. For the G2A Screen the PCR served as a template for sequencing to determine the ratio of G2G over G2A integrated construct. For the *NMT* gene a unique restriction site within the integrated construct was used for digestion to distinguish integrated G386E mutation from WT G386. Rapamycin-induced recombination between loxP sites was detected by PCR analysis of schizont-stage gDNA, harvested between 42-45 h after DMSO or rapamycin treatment. For excision PCR two primers outside the homology region and outside of the excised product were used to show that the locus of interest had

been excised, generating a smaller product than the expected PCR product under condition of non-excision.

2.2.1.8 Determination of parasitaemia by flow cytometry

Synchronized parasites were incubated with DMSO or NMTi and samples were fixed in 4% PFA, 0.2 % glutaraldehyde for one hour at 45 h PI. Subsequently samples were washed in PBS and labelled with 1:500 of 10mg/mL Hoechst 33342 (New England biolabs, Cat# 4082S) for 10min with a subsequent wash in PBS. For analysis, a Flow Cytometry CL1 Fortessa D Analyzer or Aria Fusion Sorter and Analyzer from BD with FACSDiva software v8.0.1 was used with the 450-50 filter counting 50,000 RBC per sample. Data were analysed using FlowJo LLC 2006-2015. Gating for RBCs was achieved by plots of forward scatter area against side scatter area (gate = P1). Doublet discrimination required gating on a plot of forward scatter height against forward scatter width (gate = P2) followed by a plot of side scatter height against side scatter width (gate = P3). A Hoechst stained uninfected RBC sample was used as a negative control to gate on the infected population only on a forward scanner area against UVA fluorescence with 450/50 standard filter (gate = P4). Parasitaemia was determined by the number of cells identified in gate P4 as a percentage of those in gate P3. The median fluorescence intensity (MFI) of each sample was used to determine the median number of nuclei per sample by normalizing it to the MFI of a control sample containing synchronized rings with a known MFI of one nucleus.

2.2.1.9 Analysis of parasite growth and invasion

Analysis of the growth of PfNMT[WT] and two PfNMT[G386E] parasite clones over eight generations was performed by first equalizing the starting parasitaemia (0.67 % WT, 0.73 % Clone 1 and 0.61 % Clone 2). Three biological replicates were generated in three different blood dilutions with three technical replicates each. WT parasitaemia was determined following Giemsa staining after each cycle and used to determine the dilution factor to dilute all three lines down to 0.8%. Parasitaemia was measured after each cycle using flow cytometry and Hoechst-stained parasites.

Multiple t-tests were performed for each generation (two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with false-discovery rate = 1 %).

To analyse the growth of *gap45:ha3:loxP::comp_gap45[G2A]* Clone 01 and Clone 02 as well as *gap45:ha3:loxP* and *gap45:ha3:loxP::comp_gap45*, parasites were treated with rapamycin or DMSO and adjusted to a parasitaemia of 0.1 %. At the beginning of the assay samples were taken to analyse by flow cytometry for time point 0 (see section 2.2.1.8). Each experiment was set up in triplicates: these biological replicates were achieved by three independent blood dilutions. The two clones served as an additional biological repeat. At 72 h and 120 h post invasion, samples were taken for cycle one and two during which parasites were in a late ring/early trophozoite stage, and processed as described in Section 4.2.

The invasion capacity of parasites (DMSO vs rapamycin for *gap45:ha3:loxP* / *gap45:ha3:loxP::comp_gap45* and *gap45:ha3:loxP::comp_gap45[G2A]*) as well as 3D7 parasites treated with DMSO and 140 nM of NMTi IMP-1002, was measured using synchronized mature schizonts which were Percoll purified in the subsequent cycle and then added to RBC at 1% HC at a parasitaemia of 1–3%. Samples were fixed with 4% PFA and 0.02% glutaldehyde at 0, 4, and 24 h PI enabling the percentage of newly formed ring-infected RBCs to be determined by Hoechst-staining and flow cytometry. Experiments were performed in triplicates in blood from three different donors. The data were analysed with FlowJo and GraphPad Prism to determine the standard deviation and perform a t-test for statistical significance between the samples.

2.2.1.10 Drug treatment and EC_{50} determination using the SYBR Green assay

Aliquots of 100 μ l ring-stage parasites (1–6 h post invasion [PI]) were diluted in 96-well flat bottom black plates to 0.3 % parasitaemia and 2 % haematocrit. Inhibitors were added in doubling dilutions ranging from 7.8 nM – 5 μ M for DDD85646 and 0.54 nM – 400 nM for IMP-1002 for the initial EC_{50} determination in 3D7 in a final volume of 100 μ l. For the EC_{50} of PfNMT[G386E] compared to PfNMT[WT] a range

from 0.2 nM – 10 μ M for IMP-1002 and DDD85646 as well as 0.6 nM - 40 μ M for IMP-0964 and IMP-0320 was used. The final DMSO percentage was kept at 0.05%. Cultures were incubated in gassed humidity chambers (90 % N₂, 5 % CO₂ and 5 % O₂) at 37°C for a further 96 h to increase the dynamic range and processed as described (Green et al. 2015). After 96 h, the parasites were lysed and DNA was stained by adding to each well 25 μ l lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, pH 8.0, 1.6 % Triton X-100, 0.16 % saponin, containing 0.1% SYBR Green [Life Technologies]) (final SYBR Green concentration of 0.02%) (Smilkstein et al. 2004). Plates were incubated for 1 h in the dark at RT before measurements on a FLUOStar Omega Plate reader (BMG Labtech) with excitation and emission filters of 485 nm and 520 nm, respectively. EC₅₀ values were calculated from a four-parameter logistical fit of the data using Prism software (GraphPad Software, Inc.), with subtraction of the background signal from uninfected RBC. When comparing the EC₅₀ of two parasite lines, Prism software was used to fit and constrain EC₅₀ curves at the top and bottom to improve plateau definition through a shared value of all data sets.

2.2.2 Molecular biology techniques

2.2.2.1 Molecular cloning

PCR amplification was used to generate constructs for mutant parasite transfections. KAPA® High-Fidelity DNA Polymerase (KAPA Biosystems) was used as a proof-reading enzyme for cloning according to manufacturer's instructions. For higher throughput techniques such as screening for positive parasite clones, GoTaq Green Master Mix (Promega) was used according to manufacturer's instructions. Oligonucleotide primers were used at a concentration of 0.5 μ M. If required, PCR products were purified using the QIAquickPCR Purification Kit (Qiagen).

Digestion and ligation of DNA fragments was achieved using New England Biolabs restriction enzymes and the T4 DNA Ligase kit (New England Biolabs) following manufacturer's instructions. For gel extraction to separate DNA fragments after digestion the QIAquick Gel Extraction Kit (Qiagen) was used. For transformation, *E. coli* XL10-Gold Ultracompetent Cells (Stratagene) were used following their transformation protocol. Plasmid DNA was purified from *E. coli* using the QIAprep

Spin Miniprep Kit (Qiagen) as advised by the manufacturer. DNA concentration was quantified using a nanodrop spectrophotometer (ThermoFisher Scientific). To verify the correct DNA sequence at various stages of the cloning procedure, Sanger sequencing was performed through Source Bioscience. DNA for transfection was produced in larger yields and purified through a HiSpeed Plasmid Maxi or Midi Kit (Qiagen), following the recommended protocol.

2.2.2.2 NMT gDNA amplification and sequencing from IMP-1002 resistant *P. falciparum*.

The genomic DNA sequence of the *nmt* gene in *P. falciparum* 3D7 and Dd2 is identical (www.PlasmoDB.org) and was used for primer design. Following PCR amplification, the product was sequenced and cloned into pGEM-T Easy vector after ligation of a 3'-A overhang using Amplitaq polymerase to improve the sequence quality. Sequence data were analysed using DNASTAR SeqMAN Pro and a consensus sequence for each of the parasite lines was fed into CLC Sequence Viewer 7 to create a nucleotide alignment. The nucleotide sequence was also translated into protein, which was aligned to the WT sequence using CLC Sequence Viewer 7.

2.2.2.1 Generation of repair and Cas-9 plasmid for the G2A/G2G competition screen of GAP45, ARO, TRP, CDPK1, ISP3, and S9C

For each locus a rescue plasmid was used with 200 base pair homology regions either side of the G2A mutation and guide sequence. The sequence in-between the G2A mutation and the guides was recodonised using the Codon Usage Table from PlasmoDB. The tool on the ctegd.uga.edu/ website was used to determine two guides for each target gene based on close proximity to the G2A mutation, total score as calculated by the efficiency score based on Doench et al., 2014 (Doench et al. 2014) and the CRISPRRater (Labuhn et al. 2018) (Table 2.4). Each construct was flanked by unique retractions sites (SacI and SacII) not present in any of the constructs or the pMK-RQ kanamycin resistance plasmid from Gene Art, for

linearization prior to transfection. For each construct (Table 2.5) a second plasmid was ordered containing the same homology arms but retaining the codon for glycine at position 2 with a silent mutation choosing a different glycine codon compared to the endogenous sequence.

Guide #	Primer name	Seuquence
1	G386E-guide-01_F	ATTGTTAAAATTTGGAGAAGGAGA
2	G386E-guide-01_R	AAACTCTCCTTCTCCAAATTTTAA
3	G386E-guide-02_F	ATTGTTTTAATGCCTTAGAAGTAA
4	G386E-guide-02_R	AAACTTACTTCTAAGGCATTAAAA
5	GAP45_Guide_Perrin_2018_F	ATTGAACCTCTTGAACAAGAAC
6	GAP45_Guide_Perrin_2018_R	AAACGTTCTTGTTCAAGAGGTT
7	ARO_G2A_guide-01_F	ATTGATGGGAAATAATTGCTGTGC
8	ARO_G2A_guide-01_R	AAACGCACAGCAATTATTTCCCAT
9	ARO_G2A_guide-02_F	ATTGCTTCTGCTAAGTCTTGCTCA
10	ARO_G2A_guide-02_R	AAACTGAGCAAGACTTAGCAGAAG
11	GAP45_Guide_02_F	ATTGATGTTCAAGAAGCAAAGTAA
12	GAP45_Guide_02_R	AAACTTACTTTGCTTCTTGAACAT
13	GAP45_Guide_03_R	AAACAACGTAAAGATATTGATGAA
14	GAP45_Guide_03_F	ATTGTTTCATCAATATCTTTACGTT
15	ISP3_Guide_01_F	ATTGTTTCAGGACAATGAGATATA
16	ISP3_Guide_01_R	AAACTATATCTCATTGTCCTGAAA
17	ISP3_Guide_02_F	ATTGTAAGAGTGGAATAGTATAA
18	ISP3_Guide_02_R	AAACTTATACTATTTCCACTCTTA
19	S9C_Guide_01_R	AAACACTAATCTTCAGACCGCACC
20	S9C_Guide_01_F	ATTGGGTGCGGTCTGAAGATTAGT
21	S9C_Guide_02_R	AAACCACCCACCTAGCTATTCAAA
22	S9C_Guide_02_F	ATTGTTTGAATAGCTAGGTGGGTG
23	TRP_Guide_01_F	ATTGTGCATTGCGAAGTAAGAATT
24	TRP_Guide_01_R	AAACAATTCTTACTTCCGAATGCA
25	TRP_Guide_02_F	ATTGTTATGCTAGCATGAAATTGT
26	TRP_Guide_02_R	AAACACAATTTTCATGCTAGCATAA
27	CDPK1_Guide_01_F	ATTGAGGAGAAGTAAATTTACGAA
28	CDPK1_Guide_01_R	AAACTTCGTAAATTTACTTCTCCT
29	CDPK1_Guide_02_F	ATTGTTACGAATGGAAATAATTAT
30	CDPK1_Guide_02_R	AAACATAATTATTTCCATTCGTAA

Table 2.4 CRISPR-Cas9 guide sequences used in this thesis.

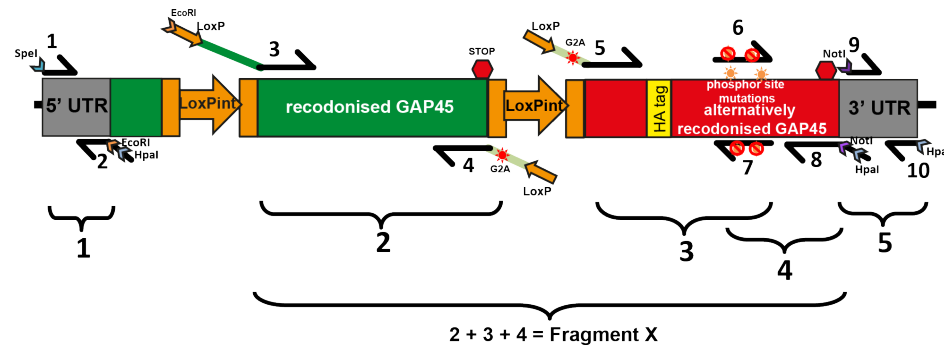
Construct/Plasmid name	target gene	Purpose	Origin of Plasmid
<i>nmt</i> [G386E]_01	<i>nmt</i>	Glycine to Alanine substitution in PNMT repair plasmid for guide 1	Gene Art
<i>nmt</i> [G386E]_02	<i>nmt</i>	Glycine to Alanine substitution in PNMT repair plasmid for guide 2	Gene Art
pDC2-cam-Cas9-U6-hDHFRyFCU	<i>cas9</i>	plasmid for guide RNA containing Cas9	Ellen Knuepfer
<i>gap45:loxP::gap45</i> [G2A]; <i>ha3</i>	<i>gap45</i>	conditional Glycine to Alanine substitution	adapted from <i>gap45:ha3:loxP</i> through cloning
<i>gap45:loxP::gap45</i> [G2G]; <i>ha3</i>	<i>gap45</i>		adapted from <i>gap45:ha3:loxP</i> through cloning
<i>aro:loxP::aro</i> [G2G]	<i>aro</i>		adapted from <i>aro:loxP::aro</i> [G2A] through cloning
<i>aro:loxP::aro</i> [G2G]; <i>ha3</i>	<i>aro</i>	conditional Glycine to Alanine substitution (construct with HA tag and with STOP codon in second copy)	Gene Art
<i>aro:loxP::aro</i> [G2A]	<i>aro</i>	conditional knock out of PfGAP45	adapted from <i>aro:loxP::aro</i> [G2A] through cloning
<i>aro:loxP::aro</i> [G2A]; <i>ha3</i>	<i>aro</i>		Abigail Perrin in Michael Blackman's Lab
<i>gap45:ha3:loxP</i>	<i>gap45</i>		Abigail Perrin in Michael Blackman's Lab
<i>gap45:ha3:loxP::comp_gap45</i>	<i>gap45</i>	complementation with WT GAP45 of conditional knock out	Abigail Perrin in Michael Blackman's Lab
<i>gap45:ha3:loxP::comp_gap45</i> [G2A]	<i>gap45</i>	complementation with GAP45[G386E] of conditional knock out	adapted from <i>gap45:ha3:loxP::comp</i> through cloning
<i>pfgap45</i> [G2G]	<i>gap45</i>	G2A/G2 competition screen	Gene Art
<i>pfgap45</i> [G2A]	<i>gap45</i>		
<i>pfaro</i> [G2G]	<i>aro</i>		
<i>pfaro</i> [G2A]	<i>aro</i>		
<i>pftrp</i> [G2G]	<i>trp</i> (PF3D7_0631000)		
<i>pftrp</i> [G2A]	<i>trp</i> (PF3D7_0631000)		
<i>pfcdpk1</i> [G2G]	<i>cdpk1</i>		
<i>pfcdpk1</i> [G2A]	<i>cdpk1</i>		
<i>pfisp3</i> [G2G]	<i>isp3</i>		
<i>pfisp3</i> [G2A]	<i>isp3</i>		
<i>pfis9c</i> [G2G]	<i>s9c</i>		
<i>pfis9c</i> [G2A]	<i>s9c</i>		

Table 2.5 List of constructs/plasmids used in this thesis.

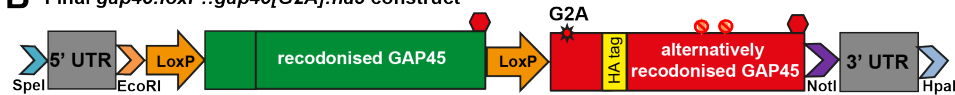
Indicated is the target gene, the purpose for the design of the construct and the origin of the synthetic construct.

2.2.2.2 Generation of repair and Cas-9 plasmid for the conditional *gap45*[G2A] and *aro*[G2A] parasite lines

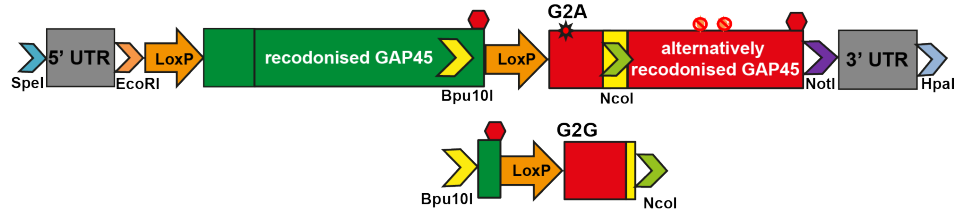
A Cloning strategy



B Final *gap45::loxP::gap45*[G2A]:*ha3* construct



C Generation of *gap45::loxP::gap45*[G2G]:*ha3* control plasmid:



D

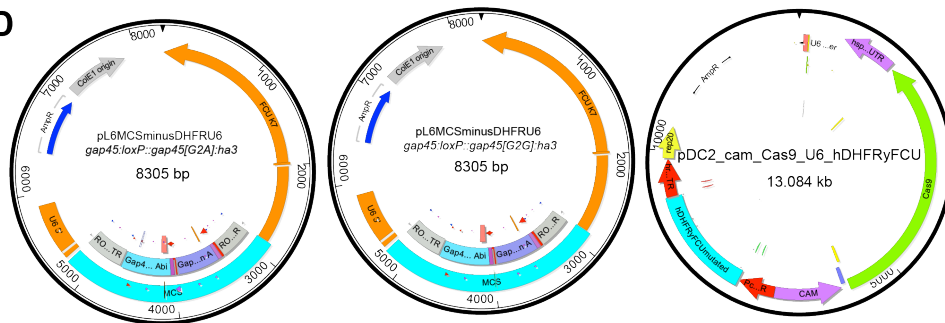


Figure 2.1 Cloning strategy for the *gap45::loxP::gap45*[G2A]:*ha3* and *gap45::loxP::gap45*[G2G]:*ha3* constructs.

A. The construct provided by Abigail Perrin in Michael Blackman's Lab designed to conditionally mutate two phosphorylation sites in GAP45 was modified to add unique restriction sites for cloning into a different vector for cloning (pL6MCSminusDHFRU6), shift the loxP sequence upstream of the ATG start codon, insert the G2A mutation into the second GAP45 copy and reverse the phosphorylation site mutation. The cloning order was the following:

- 1) Fusion PCR of PCR fragments 2, 3, and 4 with Primer 3 and 8 to generate Fragment X with EcoRI at the 5' and NotI-HpaI at the 3' end

- 2) SpeI/HpaI digest of 5' UTR with SpeI at 5' and EcoRI-HpaI at 3' and ligation into cloning vector
 - 3) EcoRI/HpaI digest of Fragment X and vector/5'UTR
 - 4) NotI/HpaI digest of 3' UTR with NotI at 5' and HpaI at 3' ends, and ligation into vector/Fragment/5'UTR
- B. The final construct contains a 5' UTR homology region, a loxP upstream of the ATG start codon, the first wild type recodonised *gap45* gene, followed by a second loxP and the second mutated (G2A), *gap45* gene.
- C. Unique restriction sites Bpu10I and NcoI, which are in close proximity to the G2A mutation, were used to exchange mutate G2A back to G2G through PCR amplification, followed by digestion and ligation into the "G2A" construct.
- D. The two final repair constructs: *gap45::loxP::gap45[G2A]:ha3* and *gap45::loxP::gap45[G2G]:ha3* as well as the Cas/guide plasmid.

The *gap45::loxP::gap45[G2A]:ha3* construct was generated using a construct for a conditional GAP45 phosphorylation site mutant (unpublished) provided by Abigail Perrin in Michael Blackman's Lab (Figure 2.1). The construct was modified through molecular cloning using fusion PCR of three PCR fragments as well as the addition of 5' and 3' UTRs serving as homology regions (Figure 2.1 A). Each step involved PCR, digestion, ligation, transformation, selection of bacterial clones, DNA extraction through mini prep, and sequencing (Figure 2.1 A). As a vector for cloning the pL6MCSminusDHFRU6 provided by Ellen Knuepfer was used. The construct contains a LoxP in front of the ATG followed by a recodonised copy of the endogenous GAP45 gene, followed by a second LoxP site with a subsequent copy of a G2A mutated GAP45 (Figure 2.1 B). The final construct was used to generate a control *gap45::loxP::gap45[G2G]:ha3* plasmid which contained two copies of GAP45 both with an N-terminal glycine (Figure 2.1 C). This was done using two oligonucleotide primer utilizing the unique restriction sites in close proximity to G2, Bpu10I and NcoI (Figure 2.1 C, Table 2.1) The guide was provided by Abigail Perrin on a pDC2-cam-Cas9-U6-hDHFRyFCU-plasmid (Knuepfer et al. 2017; Lim et al. 2016; Macpherson & Scherf 2015) and has previously been successfully used to generate a Δ GAP45 line (Perrin et al. 2018). This led to three final plasmids (Figure 2.1 D, Table 2.5). Both plasmids were transfected twice using the same guide. For linearization of the rescue plasmid, restriction enzymes PstI (transfection #1) and Scal (transfection # 2) were used, which cut 2004bp (PstI) and 1323 bp (Scal) away from the homology arms. The pDC2-cam-Cas9-U6-hDHFRyFCU-plasmid (Table 2.5) containing the guide and rescue plasmid were paired and ethanol precipitated prior transfection and dissolved in 10 μ l sterile 10 mM Tris, 1 mM EDTA (TE) buffer.

A

PF3D7_0414900 armadillo-domain containing rhoptry protein (ARO) genomic locus on Chromosome 4:

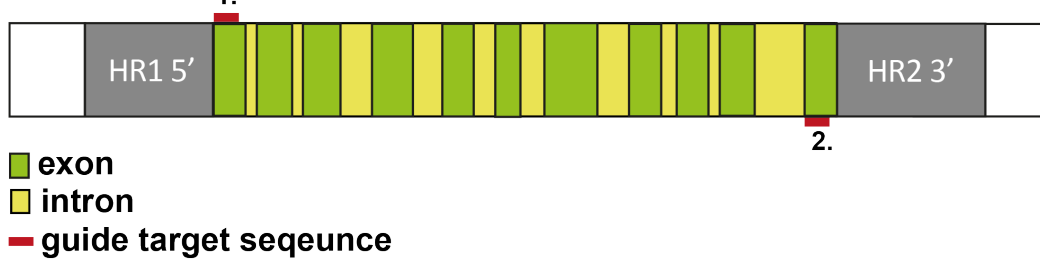
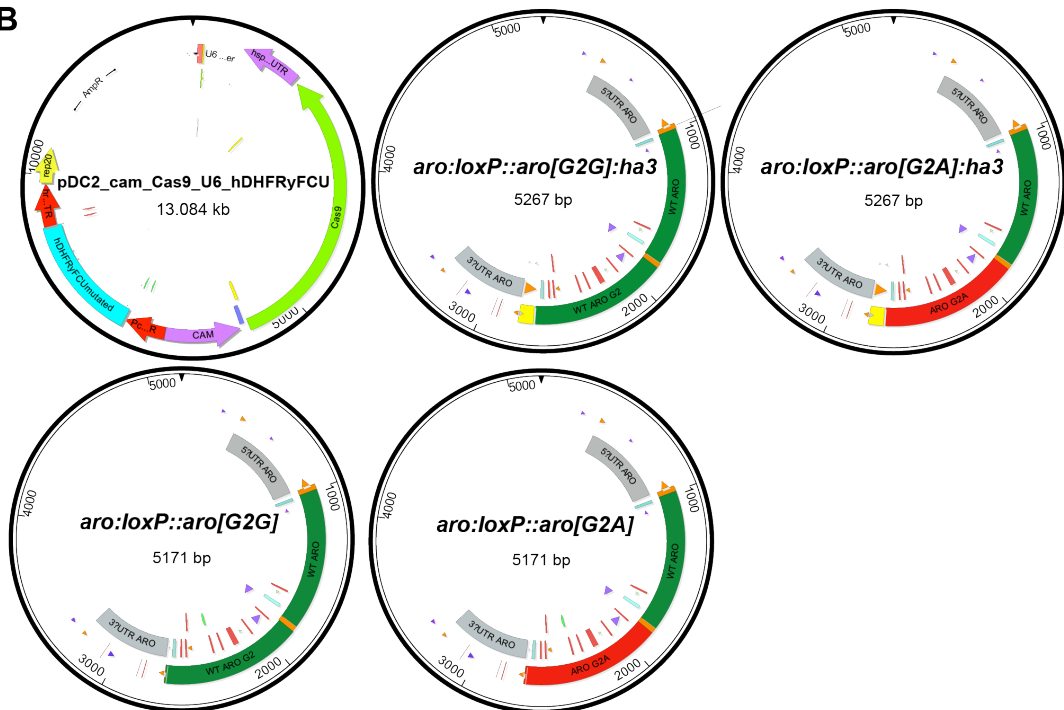
**B**

Figure 2.2 Constructs for the conditional ARO[G2A] mutant.

A. Genomic sequence of ARO showing the 11 exons and 10 introns. The location of the two guides used for the transfections are marked in red. B. Plasmid maps of the Cas9/Guide plasmid as well as the four different repair plasmids. The *aro:loxP::aro[G2A]* plasmid was ordered through geneArt. The 5' and 3' UTRs were added through PCR amplification, digestion and ligation. The *aro:loxP::aro[G2G]* was generated through PCR amplification, digestion and ligation using unique restriction sites (StuI and TfiI) around the codon encoding the N-terminal glycine. The second *aro* gene copy either terminates on a stop codon or with a triple HA-tag followed by a stop codon, which were added through PCR amplification, digest and ligation using the MfeI and XmaI restriction sites (see text for details and oligonucleotide table 2.1). The XmaI-HA3-STOP-XmaI fragment was provided by Ellen Knuefer.

The *aro:loxP::aro[G2A]* and *aro:loxP::aro[G2G]* plasmids were created with the same final layout identical to *gap45:loxP::gap45[G2A]:ha3* but the sequence in-between

the two homology regions was synthesised by Gene Art and provided on a pMK-RQ kanamycin resistance plasmid. For both ARO copies the 10 introns were spliced out by using the cDNA (Figure 2.2 A). Furthermore, the open reading frame was recodonised using the Codon Optimization tool from idtdna by converting it to the *E. coli* K12 strain and back to *P. falciparum*. The second copy was recodonised to minimise overlap to the first copy upstream and the endogenous wild type locus. The alignment function of the CLC Sequence viewer was used to determine that all three sequences (two recodonised sequences and the endogenous locus) have sufficient dissimilarity. The two guides were designed using both cas-offinder on protospacer.com as well as ctegd.uga.edu/ (Table 2.4) (relative location highlighted Figure 2.2). The construct was generated missing the stop codon after the second ARO copy and ending on an XmaI restriction enzyme site which was used to either clone in a stop codon or an HA-tag STOP. The two homology arms at the 5' and 3' UTR with 583 base pairs (5' UTR) and 556 base pairs (3' UTR) were amplified by PCR and cloned into the pMK-RQ kanamycin resistance plasmid containing the *aro:loxP::aro[G2A]* construct with SacII and SacI (5' UTR) and XmaI and PvuI (3' UTR). Prior to addition of the stop codon versus HA3-STOP, the *aro:loxP::aro[G2G]* construct was generated by using the unique restriction sites StuI and TfiI around the G2A mutation to revert the GCA (Alanine) back to GGT (Glycine). With both the *aro:loxP::aro[G2A]* and the *aro:loxP::aro[G2G]* flanked by homology regions, a STOP version was produced through PCR with oligos ARO_MfeI_STOP_F and ARO_XmaI_STOP_R (Table 2.1) inserting a stop codon after the second ARO copy. The alternative version was a digest with XmaI to insert an "XmaI-HA3-STOP-XmaI" fragment provided by Ellen Knuepfer. This resulted in four constructs: *aro:loxP::aro[G2G]*, *aro:loxP::aro[G2G]:ha3*, *aro:loxP::aro[G2A]* and *aro:loxP::aro[G2A]:ha3* (Figure 2.2 B, Table 2.5).

2.2.2.3 Generation of repair and Cas-9 plasmid for the GAP45:HA3:loxP::GAP45[G2A]comp parasite line

The *gap45:ha3:loxP::comp_gap45* construct published in (Perrin et al. 2018) and provided by Abigail Perrin comprises of a cassette of the endogenous *gap45* coding sequence and approximately 1 kb of the *gap45* 5' UTR flanking sequence, which is

believed to contain the promoter of GAP45 and was cloned upstream of the *P. berghei* dihydrofolate reductase (*PbDT*) 3'UTR. The whole construct is flanked by two homology arms targeting it to the *pfs47* locus. The G2A mutation in the *gap45:ha3:loxP::comp_gap45* generating *gap45:ha3:loxP::comp_gap45[G2A]* was achieved by a fusion PCR of two PCR fragments using primers GAP45_AJP37_SnaBI_F, GAP45_AJP37_AflIII_R, GAP45_AJP37_G2A_F, and GAP45_AJP37_G2A_R (Table 2.1). This generates a 919 base pair fragment which could be digested into the *gap45:ha3:loxP::comp_gap45* construct using restriction enzyme sites SnaBI and AflIII. The sgRNA targeting *pfs47* used has previously been published (Knuepfer et al. 2017).

2.2.2.4 Generation of repair and Cas-9 plasmid for the *PfNMT*[G386E] parasite line

The *NMT_G386E* repair plasmid mutation was synthesized using Gene Art and provided on a pMK-RQ kanamycin resistance plasmid (Figure 2.3, Table 2.5). Construct *PfNMT_G386E_01* comprises of stretches of 200 base pairs of homology region flanking the repair sequence consisting of the guide sequence ending with the G386E mutation changing the codon for glycine (GGA) to glutamate (GAA) and appropriate shield mutation destroying the guide sequence (Figure 2.3) (see also Figure 5.2 in section 5.2). Construct *PfNMT_G386E_02* designed for the second guide contains a stretch 45 base pairs between the guide sequence and the G386E mutation which has been recodonised using the *P. falciparum* Codon Usage Table from PlasmoDB. The two guides were designed using cas-offinder on protospacer.com and cloned into the pDC2-cam-Cas9-U6-hDHFRyFCU-plasmid (Knuepfer et al. 2017; Lim et al. 2016; Macpherson & Scherf 2015).

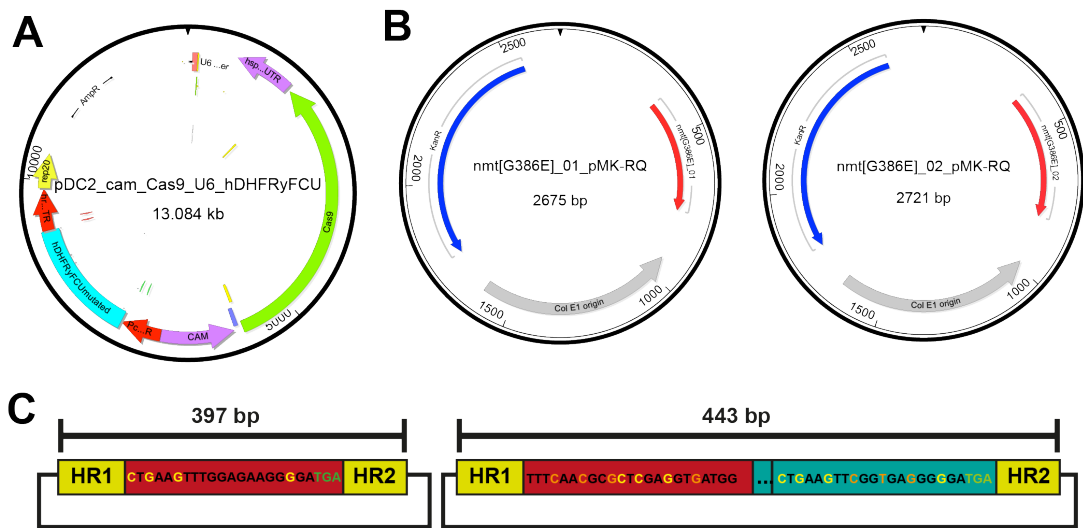


Figure 2.3 Plasmids for the PfNMT[G386E] mutant parasite line.

A. Map of the Cas9/guide plasmid. B. Map of the two repair plasmids designed with homology regions repairing the double strand break of guide 1 and guide 2. C. Sequence of repair plasmid indicating homology region in yellow, guide sequence in red and recodonised region in green. Silent point mutations for the generation of unique restriction sites in the integrated construct are highlighted in yellow. Additional silent mutations are highlighted in orange.

2.2.2.5 Digestion of repair construct and precipitation with Cas9-Plasmid

For each transfection a 5:1 molar ratio of rescue to Cas9/guide plasmid with 20 µg of Cas9 plasmid was used. Each repair plasmid was linearized either at one unique restriction site in the case of *NMT_G386E* or two unique restriction sites flanking the two homology arms, in the case of *gap45[G2A]::LoxP*, *gAP45:LoxP:G2G*, *aro:LoxP:G2A*, *aro:LoxP:G2G*, *gap45:LoP:CompG2A*, and the constructs for the G2A screen. For the G2A screen the repair constructs containing the G2G and G2A codons were added at a 50:50 ratio and digested together. After a 3 h digest period at the appropriate temperature for the restriction enzyme used, successful digest was checked on an agarose gel and enzymes were heat inactivated. This was followed by DNA precipitation. For this 2.5 volume of cold 100% ethanol were added, mixing the tube by flicking. 20 µg of guide was added followed by 1/10 of the volume of sodium acetate pH 5.2 and mixing by flicking. The DNA was either left at -20 °C overnight or for at least 1 h at -80 °C. The pellet was spun down at maximum speed for 20 min at 4 °C followed by a wash with 1 ml of 70 % ethanol. The wash was taken

off inside the hood and pellets were left to air dry. The pellet was resuspended in 10 µl TE buffer (10 mM Tris-HCL pH 8, 1mM EDTA).

2.2.2.6 Illumina® MiSeq

The G2A Screen was analysed by Illumina MiSeq. The Illumina® MiSeq provides a platform for ultra-deep end-to-end targeted sequencing of PCR products to analyse genetic variation. For this genomic DNA was extracted (see section 2.2.1.3) and specific integration selective primers containing a MiSeq adapter sequence (see below) were used to amplify a 289-466 bp fragment (depending on construct) around the codon encoding G2A/G2G (Figure 2.4, Table 2.1).

Primer overhang adapter sequence:

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus specific sequence]

Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus specific sequence]

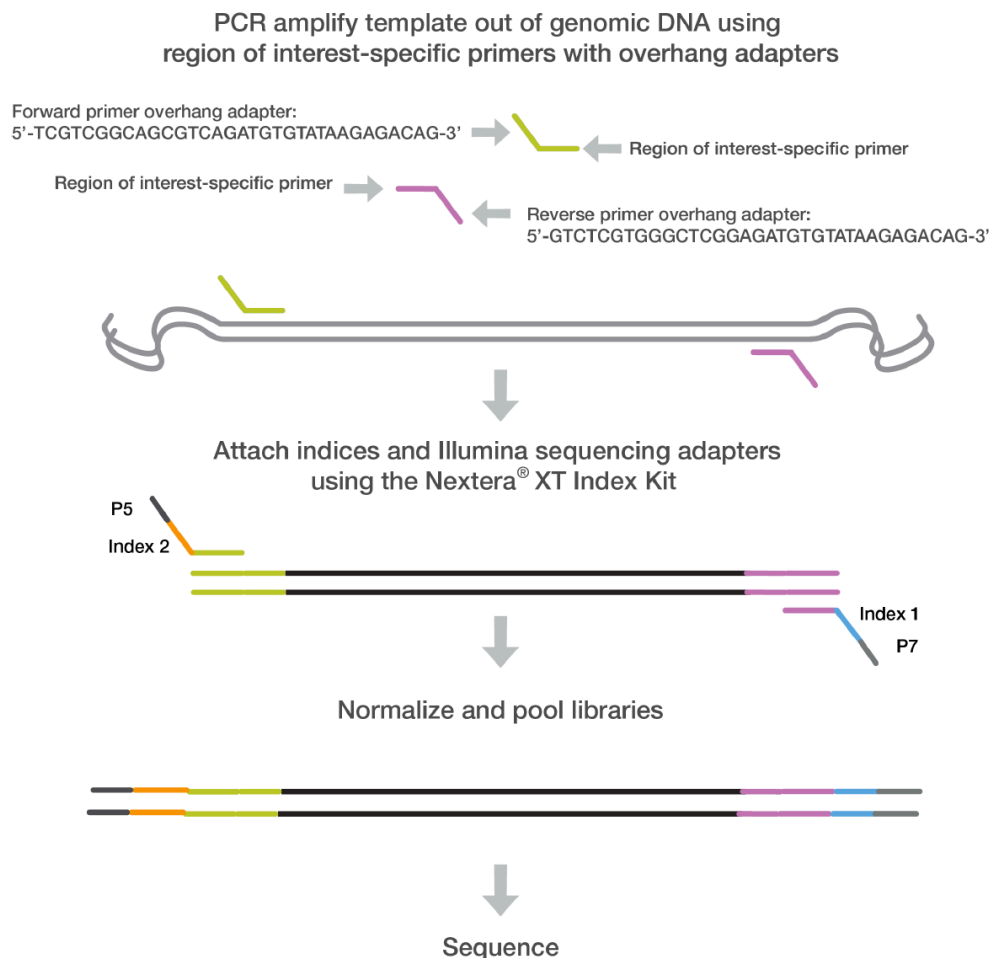


Figure 2.4 Illumina sequencing adapter addition.

Adapter overhang addition upstream and downstream of the region of interest through PCR. A subsequent PCR cycle is performed to add multiplexing induces and Illumina sequencing adapters. The figure is taken from the “16S Metagenomic Sequencing Library Preparation Guide”.

The PCR was performed under the following conditions: 3 min initial denaturation, 30 cycle of 30 sec denaturation, 30 sec annealing, and 45 sec extension, final extension 5 min using the KAPA polymerase. This was followed by a PCR clean-up using KAPA Pure beads to purify the amplicons away from the free primer and primer dimer species using a ratio of beads-to-sample of 0.8x. Beads were pipetted up and down 10x and samples were incubated for 5min at room temperature (RT). This was followed by removing the supernatant through a magnetic rack and washing the samples twice with 10x the bead volume of freshly prepared 80% ethanol leaving the sample on the magnetic rack. Samples were air-dried for 10 min and eluted in 50 µl of 10mM Tris pH 8.5 for 2 min at RT. The following steps were performed by Matthew Winder and Robert Goldstone in the Genetic Modification Service at the Crick. A 2nd stage PCR using primers specific to the added amplicon sequence attached an Illumina sequencing adapter using the Nextera® XT Index Kit. To discriminate between samples, the adapter-ligated fragments are labelled by indexing PCR with two indices at each end creating a unique barcode for each sample. This step also attaches the attachment site for the MiSeq instrument (P5 or P7) (Figure 2.4). Libraries were normalized and pooled after fluorometric quantification using a dsDNA dye, and then submitted for MiSeq. The two-step PCR pipeline generates ready-to-sequence amplicons from genomic DNA from the reverse and forward primer. In an optimally prepared library this will yield 250,000 to 300,000 sequence reads per sample depending on the instrument used. The generated sequencing data was then de-multiplexed and collapsed by the Bioinformatics and Biostatistics Service and data were analysing using DNASTAR. Robert Goldstone searched the amplicons of each sequenced genomic DNA for a nine bp DNA sequence from the sequence of the target gene specific forward and reverse primer to only selected sequence which is specific to the gene of interest. Within those sequences he searched for a nine bp DNA sequence around the codon encoding the glycine or the alanine to determine a ratio of integrated G2A versus G2G. Because of the very low number of sequence reads generated, the KAPA HyperPrep kit was used according to the manufacturer’s instructions. This was to attach the Illumina sequence adapters to increase the

attachment frequency compared to PCR. First the short overhangs of 5'-phosphates and 3'-hydroxyl groups of the PCR product were repaired to create blunt ends and then A-tailing was used to add dA-tails to the 3' of the PCR product. This creates binding sites for the Illumina-compatible adapters which can be ligated onto to the sequence instead of using the adapter tag that was attached during the first PCR. The remaining protocol was the same as describe previously.

2.2.3 Protein and immunochemistry techniques

2.2.3.1 Indirect immunofluorescence assay (IFA).

For indirect immunofluorescence assay (IFA), slides were air dried, fixed in 4 % paraformaldehyde (PFA) in PBS for 10–20 min, permeabilized in 0.1 % Triton X100 in PBS for 10 min, and blocked with 3 % bovine serum albumin (BSA) in PBS for at least 30 min at 4°C. Slides were subsequently probed with the necessary dilution of the appropriate primary antibody (Table 2.2) in a humidified chamber at RT for 1 h before washed three times in PBS. Slides were than probed with required Alexa Fluor 488 or 594-conjugate secondary antibody at the appropriate dilution for 1 h at RT (Table 2.3) followed by three washes in PBS. Slides were mounted in ProLong® Gold Antifade mounting medium containing DAPI (4',6-diamidino-2-phenylindole), and viewed on a Nikon Eclipse Ni-E imaging system with a Hamamatsu Orca-flash 4.0 digital camera and a Plan apo λ 100x/1.45 oil immersion objective. Images were captured using Nikon's NIS-Elements imaging software generating Z-stack images of individual parasites, using deconvolution options and exporting the image as a tiff file. Alternatively, images were processed using Fiji (J. Schindelin et al. 2012). Identical exposure conditions were used for each wavelength in treated (rapamycin or NMTi) and DMSO control.

2.2.3.2 Immunoblot analysis

Proteins separated by SDS-PAGE were transferred from the polyacrylamide gel to a nitrocellulose membrane using the iBLOT Transfer system from ThermoFisher Scientific. Following blocking overnight at 4°C in 5 % Milk in 0.05% Tween20 in PBS (PBS-T), membranes were incubated with primary antibody (Table 2.2) for 1 hr at

RT in 5 % milk in PBS-T, followed by three 5 min washes in PBS-T and a subsequent incubation with the species-specific secondary antibody (goat-anti-rabbit/rat/mouse IgG-HRP, Invitrogen 1:2500) (Table 2.3) for 1 h in 5 % milk in PBS-T. After a final three times 5 min washes, the membrane was incubated with either 1 ml of Amersham ECL substrate Western blotting Detection reagent (GE Healthcare lot# 9622301) or for higher sensitivity BioRad Clarity Western ECL substrate (Cat # 170-5060) was used according to the manufacturer's instructions. The signal was visualized on a ChemiDoc MP BIO-RAD Imaging System.

2.2.3.3 Cloning, expression and purification of PvNMT

The purification method has been adapted from earlier work (Bryan et al. 2011; Choi et al. 2011). Small scale expression and purification was performed by myself at the Francis Crick Institute while the large-scale purification for crystallography was done by Alexandra Reers at the Center for Global Infectious Disease Research, Seattle Children's Research Institute in Seattle. A region of the PvNMT gene encoding residues 27-410 and tagged at the N-terminus with a hexaHis sequence was cloned into a pET11a expression vector. The G386E mutation was generated using QuickChange II mutagenesis by exchanging base pairs 1079 and 1080 of the PvNMT open reading frame from GC to AA, which changes glycine at position 386 to glutamic acid. The constructs were confirmed by sequencing and the plasmid DNAs were used to transform Rosetta-gami (DE3) *E. coli* competent cells, using ampicillin for pET11a selection and chloramphenicol for the pRARE2 plasmid present in the Rosetta-gami DE3 cells to supply the tRNAs for rare codons used to codon-optimize PvNMT.

PvNMT[WT] and PvNMT[G386E] were expressed following inoculation of a single colony overnight at 37°C in 5 mL Lysogeny Broth. The PvNMT[WT] protein was expressed in 8 x 500 mL auto-induction medium (92 % ZY, 0.1 % 1M MgSO₄, 0.1 % 1000x Trace Metals, 5 % 20xNPS, 2 % 50x 5052) (2x 500mL for small scale) and the PvNMT[G386E] protein in 19 L due to low yield. Auto-induction medium was combined with appropriate antibiotics and incubated with 1:100 dilution factor from the overnight culture at 37 °C for 4 h. Temperature was then reduced to 18°C for

18-22 h. Bacterial cells were harvested by centrifugation and pellets from 4 L (for the WT protein) re-suspended in 180 mL of lysis buffer (20 mM HEPES, 5 % glycerol, 10 mM imidazole, 0.5 % CHAPS, 21 mM MgCl_2 , 1 mM dithiothreitol (DTT) and Complete Protease Inhibitor Cocktail EDTA-free). For the PvNMT[G386E] protein cells from the 15 L were resuspended in 2x 300 mL, sonicated separately and then combined prior to application to the column. They were lysed using a BRANSON 550 sonication probe (20 % strength, 5 sec on 10 sec off for 10 min, 15 min total of sonication). The lysate was cleared by centrifugation at 48,384g RCF for 60 min at 4°C in a 25.50 JA rotor.

For large scale preparations the lysate was filtered through a 0.45 μM syringe filter and applied to a HisTRAP FF 5 mL column at 4°C and eluted with a linear gradient over 15 column volumes with 20 mM HEPES, 0.3 M NaCl, 5 % glycerol, 0.5 M imidazole; pH 7.0. Fractions containing the target protein were pooled and concentrated to about 2-3 mL using 10 kDa-cut off Pall centrifuge concentrators.

For small scale preps 2x 500 ml (PvNMT[WT]) and 4 x 500 ml (PvNMT[G386E]) were inoculated overnight and lysed in 180 ml (PvNMT[WT]) or 360 ml (PvNMT[G386E]) of lysis buffer and sonicated with a sonication probe (20% strength, 5sec on 10sec off for 10min (PvNMT[G386E]) or 5min (PvNMT[WT])). The lysate was incubated in batch with 20 mL of Ni-NTA Agarose beads (QIAGEN) equilibrated with lysis buffer in a 500 mL Duran bottle at 4°C for 1 h. The beads were washed using a re-usable glass Fritted Funnel column and a pressure pump with 20x bead volume of wash buffer using a pressure pump (20 mM HEPES, 5 % glycerol, 30 mM imidazole, 0.5 % CHAPS, 21 mM MgCl_2). The bound protein was eluted from the beads using 3x bead volume of elution buffer through gravity (0.3 M NaCl, 20 mM HEPES, 5 % glycerol, 0.5 M imidazole; pH 7.0). Eluted proteins were analyzed using instant blue (Expedeon Prod # ISB1L). Nickel elution was filtered and concentrated using Centricon Plus 70 with a 30kDa cut-off.

For both small and large scale preparations concentrated protein was diluted 100-fold with anion exchange buffer (20 mM Tris-HCl, 1 mM TCEP; pH 8.9) and applied directly to a HiTRAP Q HP 5 mL column (1 mL column for small scale) at 2 mL/min. Unbound sample was washed away with 5-8 column volumes (CV) at

2 mL/min for the 1 mL column and 3 mL/min for the 5mL column and eluted with 2 %-50 % of the elution buffer over 15 CV at 0.5 mL/min for the 1 mL column and 1 mL/min for the 5mL column. The 2 mL fractions (5 mL column) or 0.5 mL fractions (1 mL column) were analysed by applying 10 μ L to an SDS PAGE gel and visualized using instant blue. For protein for crystallography, the His tag was cleaved from the PvNMT[WT] and PvNMT[G386E] proteins via 3C protease (His-MBP-3C) at a 1:50 protease: protein ratio overnight at 4°C in dialysis buffer (25 mM HEPES pH 7.5, 500 mM NaCl, 5 % (v/v) glycerol, 1 mM TCEP, and 0.025 % sodium azide). NMT was recovered by applying the dialysate to a second Immobilized metal affinity chromatography (IMAC) column. Fractions containing target protein were pooled and concentrated to 70 μ L for the small scale or 4 mL for large scale and loaded onto Superdex 75 10/300 or 26/600 columns and eluted with size exclusion buffer (0.3 M NaCl, 20 mM HEPES, 5 % (v/v) glycerol, 1 mM TCEP; pH 7.0). Fractions were analyzed on an SDS-PAGE gel and fractions containing the target protein were either pooled in the case of PvNMT[WT] or because of limited material kept separate for the PvNMT[G386E] protein. Fractions were concentrated and flash frozen and stored at – 80°C until further use.

For small scale expression the purified protein was detected by Western blotting, using the anti-PvNMT antibody (PvNMT protein made by Jim Brannigan at York University and antibodies raised by David Moss, MRC National Institute for Medical Research). For Western blots, proteins were transferred from the gel to nitrocellulose membrane with the iBlot™ Transfer System. Following blocking overnight at 4°C, membranes were incubated with primary antibody for 1 h at RT in blocking solution and incubated with secondary antibody (goat-anti-rabbit/rat/mouse IgG-HRP, Invitrogen 1:2500) for 1 h in blocking solution. Detection was carried out using GE Healthcare ECL Western blotting detection reagents or BioRad Clarity Western ECL Substrate and visualized on a ChemiDoc MP BIO-RAD Imaging System.

2.2.3.4 CPM-based PvNMT IC₅₀ determination

To measure the activity of the purified PvNMT[WT] and PvNMT[G386E], an assay was used with thiol-selective fluorescent dye (7-diethylamino-3-(4-maleimido-

phenyl)-4-methylcoumarin (CPM)) (Sigma Aldrich, Cat# C1484) to monitor the formation of coenzyme A. The assay was performed as published in (Goncalves, Brannigan, Thinon, et al. 2012). Reagents were prepared in 20 mM sodium phosphate, 0.5 mM EDTA, 0.1 % Triton X-100 at pH 7.90-7.95 with the corresponding DMSO concentrations. First, 10 μ L of a 10 % DMSO/water (v/v) solution containing a dilution series of NMT inhibitor, 50 μ L of NMT in 1 % DMSO (final concentration of 6.3 nM), and 25 μ L of myristoyl-CoA solution in 1 % DMSO (4 μ M final concentration), were combined in Black microplate 96-well non-sterile flat bottom polypropylene plates (655209). The reaction was started by adding 25 μ L of CPM and a 15-residue HsSrc peptide solution in 5 % DMSO (final concentration CMP 8 μ M, HsSrc peptide 4 μ M). For the continuous assay the fluorescence intensity was monitored over 45 min at 0.5-min intervals (excitation at 380 nm, emission at 470 nm) at 25°C using the EnVision Xcite Plate Reader (exported with EnVision Workstation version 1.13.3009.1409). For the quenched assay the reaction was stopped after 30 min, at which point the initial reaction rate was still in the linear range, by adding 60 μ L of the quench solution (50 mM acetic acid, 50 mM sodium acetate, pH 4.74-4.78).

2.2.3.5 Thermal shift assay

The thermal shift assay was performed by Adam Flinders at GlaxoSmithKline, Stevenage. Purified recombinant PvNMT[WT] and PvNMT[G386E] were diluted to 0.1 mg/mL in PBS buffer containing 10% glycerol and 1:1000 dilution of Sypro Orange in the presence or absence of 4 μ M myristoylCoA. Test compounds were diluted to 500 μ M in DMSO, and 0.5 μ L of diluted compound was added to 49 μ L of enzyme in a 96-well plate resulting in two technical replicates with a final volume of 20 μ L and 1% (final) DMSO with a compound concentration of 5 μ M. Each compound was tested twice to give two experimental replicates, each with two technical replicates. Reference thermal melting temperatures (Tms) were obtained using 1% DMSO without any compound present. The plates were subjected to a temperature gradient from 300K to 348K at a rate of 1K/min using a FluoDia fluorescent plate reader. Fluorescence data were acquired with an excitation filter of 486 nm and an emission filter at 610 nm, and the raw data were exported to Prism or Excel for analysis. Data were processed to

identify the fluorescence maxima and minima, and the midpoints of melting curves were determined by fitting to a Boltzmann equation, where T_m is the temperature corresponding to the half maximum response ($Y = y_{\min} + (y_{\max} - y_{\min}) / (1 + \exp((T_m - T)/\text{slope}))$). Results were expressed as ΔT_m in °C, where ΔT_m is the difference between T_m in the presence of compound subtracted from that in the reference without compound present.

2.2.3.6 Surface plasmon resonance (SPR) assay

The SPR assay was performed with the help of Chun-wa Chung and David Charter during a placement at GlaxoSmithKline in Stevenage. The surface plasmon resonance experiments were performed using a Biacore T200 (GE Healthcare) equipped with research-grade CM5 sensor chips. PvNMT (5.14 mg/mL, 45.0 kDa, 20 mM HEPES pH 7.0, 300 mM NaCl, 5 % glycerol, 1 mM TCEP) was diluted to 0.0514 mg/mL in 10 mM sodium acetate pH 6.0. PvNMT[G386E] (0.95 mg/mL, 47.3 kDa, 20 mM HEPES pH 7.0, 300 mM NaCl, 5 % glycerol, 1 mM TCEP) was diluted to 0.0428 mg/mL in 10 mM sodium acetate pH 6.0. Each ligand was immobilized onto separate flow cells using amine-coupling chemistry. The surfaces of all three flow cells (one empty negative control) were activated for 7 min with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide) and 0.1 M EDC (3-(N,N-dimethylamino) propyl-N-ethylcarbodiimide) at a flow rate of 10 $\mu\text{L}/\text{min}$. PvNMT was immobilized at a density of 900 - 1100 RU onto flow cell 3, and PvNMT[G386E] at 600-1700 RU onto flow cell 4. Flow cell 1 was left blank to serve as a reference surface. All three surfaces were blocked with a 7 min injection of 1 M ethanolamine, pH 8.0. IMP-0320 was used as a positive control in the assay as it has been extensively used by GSK for routine NMT SPR assays.

The analytes (IMP compounds [400-550 Da] dissolved in DMSO to a concentration of 500 μM ,) were serially diluted 1:3 in DMSO, and then 1-3 μL of each dilution was dissolved in an assay buffer of 10 mM HEPES pH 7.4, 150 mM NaCl, 0.005 % Tween 20, 4 μM myristoylCoA to a final DMSO concentration of 1 %. To collect binding affinity data, the analyte concentrations (0-0.4 μM except from DDD85646: 0-1 μM) were injected sequentially over all four flow cells at a flow rate of 30 $\mu\text{L}/\text{min}$

and at a temperature of 25°C. The complexes formed were allowed to associate for 200-700 sec and then dissociate for 600-2400 sec, dependent on the analyte kinetics observed. The surfaces were washed with an injection of 50 % DMSO between each analyte injection. The data were analyzed within Biacore T200 Evaluation Software v2.0.

Equilibrium sensorgram values were exported and the offset set to zero before fitting to a global 'One site – Specific binding' nonlinear regression model (Motulsky & Brown 2006) in GraphPad Prism 7. As differential binding of compounds to PvNMT[G386E] and PvNMT[WT] were of greatest interest, the ratio of K_D PvNMT[G386E] to that of PvNMT [WT] was calculated for each compound dose response on the same chip within an experiment.

2.2.3.7 Crystallography

Crystallography was carried out by Stephen Mayclin at Seattle Structural Genomics Center for Infectious Disease (SSGCID) in Seattle and Beryllium Discovery Corp. on Bainbridge Island. PvNMT[WT] and PvNMT[G386E] were crystallized using the sitting drop vapour diffusion method at 7.8 mg/mL and 12.2 mg/mL respectively against a focused screen generated from a published literature condition (Yu et al. 2012). Drops contained 0.4 μ L protein (supplemented with either 0.5 mM Myr-CoA or 0.5 mM Myr-CoA and 0.5 mM inhibitor) and 0.4 μ L crystallant. Drops were equilibrated against 80 μ L of the crystallant in Compact Jr crystallization trays (Rigaku Reagents) and incubated at 16°C. Large, rod-morphology crystals appeared between one and two weeks and were cryo-protected with 20-25 % (v/v) ethylene glycol supplemented with ligand(s) prior to vitrification in liquid nitrogen for X-ray data collection.

X-ray diffraction data were collected at the LS-CAT beamline 21-ID-F at the Advanced Photon Source. Collection information and processing statistics are reported in appendix Table 7.8. The data were integrated with XDS and reduced with XSCALE (Kabsch 2010). Data quality assessment and space group assignment were performed using POINTLESS (Evans 2006). Structures were solved by molecular replacement using MoRDa (Vagin & Lebedev 2015), using PvNMT chain

A (PDB accession code 4B14) in the same crystal form as a search model. The structures were refined in Phenix (Adams et al. 2010), with manual model building in Coot (Emsley & Cowtan 2004). Quality of the models was assessed with MolProbity (V. B. Chen et al. 2010). Figures were generated using Pymol (Wang et al. 2015) and CCP4MG (McNicholas et al. 2011). Inhibitor omit maps were generated by complete removal of inhibitor atoms from fully refined structures and running an additional refinement cycle in Phenix (see appendix Figure 7.1). Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 6MAY, 6MAZ, 6MB0, and 6MB1.

2.2.4 Chemical Proteomics

2.2.4.1 Metabolic tagging of parasites.

For YnMyr (using YnC12 - tetradec-13-ynoic acid) and YnPal (using YnC16 - heptadec-17-ynoic acid) tagging experiments, schizonts were purified as described before (see 2.2.1.1). Parasites at the appropriate stage (usually at 34 h PI) were used based on initial examination of Giemsa-stained thin blood smears. Parasites were labelled using 25 μ M YnMyr for the appropriate length. For YnPal labelling the YnC16 molecule was stabilized with a base and absorbed with BSA to maximize its incorporation (Thinon et al. 2018). For this the required amount for feeding (120 μ l of a 50 mM Stock of YnC16 for 240 ml RPMI) was combined with 600 μ l of 0.01M NaOH and brought to 70°C for 3-4 min. 1.5 ml of warm 5% BSA was added and solution was heated at 37°C for 3-4min. The solution was added to the appropriate volume of RPMI, filtered through a 0.2 μ m filter unit, and parasites were fed with the YnC16-containing RPMI. In all experiments, the final DMSO percentage did not exceed 0.05%. Cultures were re-incubated during the time of YnMyr labelling and NMT inhibition. Parasites cultures were pelleted and RBC lysed and proteins extracted as described below (see section 2.2.4.2).

2.2.4.2 Isolation of protein material from *P. falciparum*

Parasites of the appropriate stage were either purified through percoll, washed and pelleted or directly pelleted without purification. The pellet was lysed in 0.15 %

saponin for 10 min on ice at 1.5x the pellet volume. The pellet was washed with PBS until the supernatant was clear and was stored at -80°C. Thawed parasite pellets were extracted in 10x the pellet volume of 1 % Triton, 0.1 % SDS in PBS containing protease inhibitors (EDTA-free – as EDTA will otherwise sequester the CuSO₄ in the Click reaction) on ice for 20 min with 1 min sonication. Insoluble material was pelleted for 20 min at 17,000 g in a Heraeus Fresco 17 at 4°C and supernatant containing proteins was transferred to a separate Eppendorf tube, snap frozen and stored at -80°C. The protein concentration of the lysate was measured by Pierce™ BCA Protein Assay Kit (23225, ThermoFisher Scientific) per the manufacturer's instructions.

2.2.4.3 Copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) labelling.

Reagent	Concentration of stock	Final concentration in reaction
AzTB or AzRB2	10 mM in DMSO	50 µM
CuSO ₄	50 mM in H ₂ O	1 mM
TCEP	50 mM in H ₂ O	1 mM
TBTA	10 mM in DMSO	50 µM

Table 2.6 CuAAC reagents and concentration used for Click reaction.

Click reagents are added to the reaction in the order shown. For 100 µl of lysate, 6 µl of click reaction is used.

The protein lysate was adjusted to 1 mg/mL with PBS, premixed click reagents (100 µM Capture reagent YnTB, 1 mM CuSO₄, 1 mM TCEP, 100 µM TBTA – mixed in the order stated and incubated for 2min) (Table 2.6) were added at an

equivalent of 6 µl of the click reaction mix to 100 µl of protein solution as described in (Wright et al. 2014). Samples were subsequently vortexed for 1 h at RT and quenched by the addition of 10 mM EDTA, followed by protein precipitation with 2 volumes of methanol, 0.5 volumes of chloroform and 1 volume of water. After a 10 min spin at 13300 rpm (17,000 g) in a Heraeus Fresco 17 the top layer (MeOH/water) was removed and 0.5 ml of ice cold methanol was added prior to vortexing / sonication to break the protein disc. Samples were then spun for 10 min at 4°C at 13300 rpm (17,000 g) in a Heraeus Fresco 17 and pellets were air-dried for ~15 min. Proteins were resuspended in 2 % SDS, 10 mM DTT, in PBS and vortexed for 15-30 min or until protein disc was resuspended. Samples were diluted to 1-2 mg/ml

depending on experiment. For visualization through in gel-fluorescence, 4x samples loading buffer (NuPAGE LDS sample buffer, NP0008 Invitrogen) was combined with 2-mercaptoethanol (4 % final) or 400 mM DTT, and added to 10 µg of each sample. The samples were heated for 7 min at 95°C prior to SDS-PAGE by running samples on precast NuPAGE gels (ThermoFisher Scientific).

2.2.4.4 Base cleavage of esters of GPI-anchored proteins.

Following click reaction, precipitation, and resuspension in 2 % SDS, 10 mM DTT, in PBS and dilution to 2 mg/ml, proteins were treated with 0.2 M NaOH for 1 h at RT with vortexing. Samples were neutralized with an equivalent volume and concentration of HCL and diluted with PBS to 1 mg/ml to be analysed on a gel.

2.2.4.5 Gel-based fluorescence analysis.

Samples were separated by SDS-PAGE. In-gel fluorescence imaging was used to scan the gels with a Cy3 filter to detect the TAMRA fluorophore on the capture reagent AzTB using a Pharos FXTM Plus Molecular Imager (BIO RAD). Molecular weight markers (Precision Plus All Blue Standards, Bio-Rad) were detected with a Cy5 filter using an external laser molecular imager FX from BIO RAD.

2.2.4.6 Quantification of fluorescence signal

ImageJ was used for quantification of fluorescent bands. A thin rectangle was drawn around the band to be analysed. Using the 'Cmd M' (measure) function an intensity profile was plotted. The signal of each band of interest was measured and normalizing relative to the DMSO control. Any background from no YnMyr was subtracted. The TC₅₀ was calculated by fitting data to a log(inhibitor) vs. response – variable slope (four parameters) IC₅₀ function using Prism7.

2.2.4.7 Acyl-PEG exchange

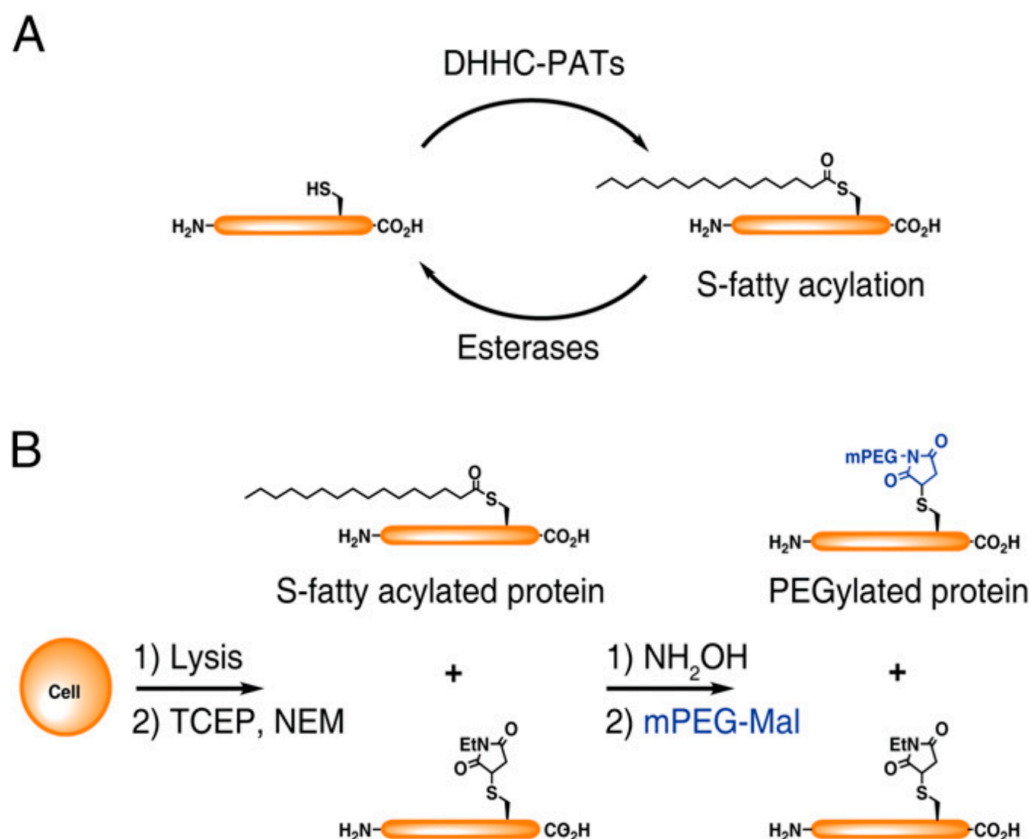


Figure 2.5 Mass-tag analysis of protein S-fatty acid acylation.

A. S-fatty acylation controls the stability, trafficking, and function of membrane proteins and is regulated by esterases and DHHC-protein acyltransferases (DHHC-PATs)

B. During acyl-PEG exchange (APE), cell lysates are reduced with Tris(2-carboxyethyl)phosphine (TCEP), and then free cysteine residues are capped with N-ethylmaleimide (NEM). S-fatty acid groups are removed by hydroxylamine (NH_2OH) treatment, and the exposed cysteines are reacted with methoxy(polyethylene glycol)-maleimide (mPEG-Mal). Proteins are separated by SDS/PAGE and analysed by Western blot, enabling the detection of both unmodified and S-fatty acylated proteins. Image taken from Percher et al., 2017

For Acyl-PEG exchange (APE), parasites lysate (in 1% Triton X-100, 0.1% SDS, EDTA-free protease inhibitor cocktail) were treated as previously described (Percher et al. 2016) (Figure 2.5). Parasite lysates were adjusted to 2 mg/ml and 92.5 μl per condition were treated to reduce Cys residues with 5 μl of 200 mM neutralized Tris(2-carboxyethyl)phosphine TCEP (Thermo) for a final concentration of 10 mM for 30 min with nutation. To block free Cys residues 2.5 μl of N-ethylmaleimide (NEM) was added (freshly prepared 1 M solution, 1/40, final concentration 25 mM). Reductive

alkylation of the proteins was then terminated by methanol-chloroform-H₂O precipitation (4:1.5:3) with sequential addition of methanol (400 µl), chloroform (150 µl), and distilled H₂O (300 µl) (all prechilled on ice). The reactions were then mixed by inversion and centrifuged (Centrifuge 5417R, Eppendorf) at 20,000 × g for 5 min at 4°C. To pellet the precipitated proteins, the aqueous layer was removed, 1 ml of prechilled MeOH was added, the Eppendorf tube inverted several times and centrifuged at 20,000 × g for 3 min at 4°C. The supernatant was then decanted, and the protein pellet washed once more with 800 µl of prechilled MeOH, centrifuged again, and dried using a speed-vacuum (Centrivap Concentrator, Labconco). To ensure complete removal of NEM from the protein pellets, the samples were resuspended with 100 µl of TEA buffer containing 4% SDS, warmed to 37 °C for 10 min, briefly (~5 s) sonicated (Ultrasonic Cleaner, VWR), and subjected to two additional rounds of methanol-chloroform-H₂O precipitations, as described above. For hydroxylamine (NH₂OH) cleavage of the palmitoylation thioester and subsequent alkylation with methoxy(polyethylene glycol)-maleimide (mPEG-Mal), the protein pellet was resuspended in 100 µl TEA buffer containing 4% SDS, 4 mM EDTA and split into two 50 µl samples. One sample was treated with 150 µl of 3 M neutralized NH₂OH dissolved in TEA buffer pH 7.3, containing 0.2% Triton X-100 (Fisher) to obtain a final concentration of 0.75 M NH₂OH. A set of control samples was not treated with NH₂OH instead diluted with 150 µl of EA buffer with 0.2% Triton X-100. Samples were incubated at RT for 1 h with nutation. The samples were then subjected to methanol-chloroform-H₂O precipitation, as described above, and resuspended in 100 µl TEA buffer containing 4% SDS, 4 mM EDTA, warmed to 37 °C for 10 min, and briefly (~5 s) sonicated and treated with 150 µl TEA buffer with 0.2% Triton X-100 and 4 mM mPEG-Mal (10 kDa; Sigma) for a final concentration of 1 mM mPEG-Mal. Samples were incubated for 2 h at RT with nutation before a final methanol-chloroform-H₂O precipitation. Again, samples were resuspended in 100 µl TEA buffer containing 4% SDS, 4 mM EDTA, warmed to 37 °C for 10 min, and briefly (~5 s) sonicated. 10 µg of each samples were added to 1x Laemmli buffer, heated for 5 min at 95 °C and run on a 3-12% Bis-Tris SDS-Page Gel and analysed by Western blot for GAP45 (Rabbit anti-GAP45 (affinity purified) 1:13.000), and CDPK1 (Rabbit anti-CDPK1 (affinity purified), 1:10.000) (Table 2.2).

2.2.4.8 Pull down of labelled proteins

After click chemistry and precipitation, proteins were diluted with PBS to 1 mg/ml. For proteomics, an agarose resin mixture was created for pull down (PD) containing 1/3 NeutrAvidin™ Agarose Resin and 2/3 Pierce Control Agarose resin (ThermoFisher Scientific) to minimize digestion of streptavidin from the beads, using a total of 30 µl of resin mixture for up to 300 µg of protein lysate. For SDS-PAGE detection, 30 µl NeutrAvidin Agarose Resin were used per 150 µg of sample. Prior to pull down, the resin mixture was washed three times with 5x the bead volume of 0.2% SDS in PBS. Beads were spun at 3000g for 2 min to pellet them and vortex-mixed for 1 min to wash them. Protein lysate was incubated with the resin for 2 h at RT on a shaking vortex at minimal speed.

For analysis by SDS PAGE, the supernatant was removed, beads were washed with three times 0.2 % SDS in PBS. Beads were eluted with 10 µl of 0.2% SDS in PBS with added 4x LDS sample buffer containing 400 mM DTT (final 100 mM) and then boiled for 10 min. Aliquots of supernatant and pre-PD sample were saved for gel analysis. Typically, 150 µg total protein was enriched in this way and the equivalent of 10 µg loaded on a gel for supernatant and pre-PD sample. After elution the sample received an additional spin and was loaded on the gel.

For proteomic analysis following pull down, samples were washed three times with 5-10 volumes of 1% SDS in 1xPBS, two times 4M Urea in 50mM Triethylammonium bicarbonate (TEAB) and an additional three times with 50mM TEAB followed by processing as described previously in (Broncel et al. 2016). TEAB was used instead of AmBic, to prevent the primary amines of Ammonium Bicarbonate (AmBic) reacting with the isobaric tagging reagent during Tandem Mass Tag (TMT) labelling. The YnMyr pull down was the only experiment where label free quantification was used, and in this case AmBic was used. To improve detection of cysteine-containing peptides, the thiols were reduced and alkylated. Protein samples were reduced with 10mM DTT in 50mM TEAB for 30min at 55°C and alkylated with 10 mM Iodoacetamide (IAA) in 50mM TEAB for 30min at RT in the dark. Overnight trypsinization with 0.12 µg trypsin gold (Promega UK Ltd, Cat. # V5280) per 300 µg of sample for pull down samples created tryptic peptides which carry at least two

positive charges on their N-terminal amine and C-terminal lysine or arginine. This improves elution and ionization of the peptides due to their charge. Trypsination was followed by treatment with 1.5 % trifluoroacetic acid (TFA) (ThermoScientific Cat. #28902) and subsequent stop-and-go extraction tips (STAGE-tips) of peptides on reverse phase C18 poly(styrenedivinylbenzene) polymer cation exchange (SDB-XC) membranes. While peptides are precipitated on the column, salt ions and detergents are removed. Samples were eluted in 79% MeCN and 21% water. The liquid content was evaporated to dryness through a speed vac concentrator.

2.2.4.9 Preparation of samples for LC-MS/MS

YnMyr pull down +/- IMP-1002:

For the YnMyr pull down of parasites treated with and without 120 nM of NMTi IMP-1002 from 34 - 45 h PI, STAGE-tipped and dried samples stored at -80°C were thawed for 5 min at RT. 15 µl of 0.5% TFA/ 2% MeCN/water were added to the peptide mixture. This was vortex-mixed for 5 min, spun down briefly, and sonicated for no more than 5 min. This process was repeated again followed by a final spin at 13300 rpm (17,000 g) in a Heraeus Fresco 17 for 10 min at 15°C. 11 µl of each sample was transferred to an autosampler compatible vial.

Whole proteomics:

For whole proteomic samples, 50 µg of protein lysate (before and after saponin treatment) were precipitated with MeOH:Cl₃CH:H₂O and washed with 200 µl methanol and spun down at 17,000 g for 10 min at 4°C. Protein pellets were solubilized with 20 µl 0.2% ProteaseMAXTM Surfactant (Promega UK Ltd, Cat. # V2071) / 50 mM TEAB for 1-2 h on a vortex with occasional sonication. Protein samples were reduced with 5 mM DTT in 50 mM TEAB for 20 min at 56°C (1 µl of 0.5M in 100 µl) and alkylated with 14.85 mM IAA (2.7 µl of 0.55M in 100 µl) in 50 mM TEAB for 30min at RT in the dark. Samples were trypsinised with 1.8 µg per 50 µg of trypsin gold (Promega UK Ltd, Cat. # V5280) at 0.05% ProteaseMAX (additional 1 µl of 1% ProteaseMAXTM Surfactant to 100 µl). After trypsinisation, the condensate was collected from the tube lid by centrifugation at 12,000–16,000 × g for 10 seconds. TFA was added at 0.5% and incubated for 5 min at RT to inactivate the

trypsin. This was followed by STAGE-tip purification on a reverse phase C18 SDB-XC membrane and elution in 70% MeCN and 30% water. The liquid content was evaporated to dryness through a speed vac concentrator.

2.2.4.10 Tandem Mass Tag (TMT) labelling and High pH Reverse Fractionation

Immediately before use, the TMT label reagents were equilibrated to RT. For 0.8 mg vials, 41 µl (5 mg vials, 256 µl was used) of anhydrous acetonitrile was added to each tube and the reagent allowed to dissolve for 5 min with occasional vortexing. The tubes were briefly centrifuged to gather the solution. For the whole proteomic experiment, the TMT10plex Label Reagent Set from ThermoFisher Scientific was used (Cat # 90309) instead of a 6-plex due to availability at the time per the manufacturer's instructions (see also Figure 3.7 in section 3.4). In brief, samples were dissolved in 25 µl 50 mM TEAB and sonicated for 10 min, 0.2 mg of TMT label reagent was added to each sample and incubated for 1 h. To quench the reaction, 8 µl of 5 % hydroxylamine were added to the sample and incubated for 15 minutes. A very small quantity (about 5%) was taken to perform a label check through an initial LC-MS/MS run to determine the ratio of labelled reporter ions. The ratio was corrected for any differences in labelling efficiency before mixing. Samples were combined into one tube at equal amounts. This was followed by high pH reverse fractionation with a gradient step wise elution from 5-50% acetonitrile to increase the proteome coverage using the kit from ThermoFisher Scientific (Cat # 84868) per the manufacturer's instructions (see also Figure 3.7 in section 3.4). In short, the high-pH, reversed-phase fractionation spin column was equilibrated with 2x acetonitrile and 2x 0.1% TFA through centrifugation. The digested sample was loaded through centrifugation and washed with LCMS-grade water, followed by a wash with 5% ACN, 0.1% TEA. This was followed by elution with 5%-50% acetonitrile to generate 9 fractions (wash with 5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25% and 50%). This stepwise elution, known as fractionation, from the STAGE-tips with increasing acetonitrile concentration allows additional separation of the peptides prior to LC/MS. The liquid content of each sample was evaporated to dryness through a speed vac

concentrator. Peptide extract mixtures was re-suspended in 15 µl 0.1 % TFA, with 10 µl per injection.

2.2.5 Proteomic data analysis

2.2.5.1 LC-MS/MS analysis

For the YnMyr pull down +/- 120 nM IMP-1002, mass spectrometry data were acquired on a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer. The Q Exactive was equipped with a nano-LC system, an electrospray ionization injector, a quadrupole, a C-trap to retain ions, an higher energy collision dissociation (HCD) fragmentation chamber and an orbitrap analyser. The analysis was performed using an EASY-Spray™ LC column with a particle size of 2 µm and 50 cm x 75 mm inner diameter (Thermo Fisher Scientific) using a 2 h MeCN gradient in 0.1% aqueous formic acid at a flow rate of 250 nl/min. Easy nLC-1000 was coupled to a Q Exactive mass spectrometer via an easy-spray source (Thermo Fisher Scientific). The Q Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 70,000 at m/z 350-1650. Up to 10 of the most abundant isotope patterns with charge 2+ or higher from the survey scan were selected with an isolation window of 2.0 m/z and fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 17,500 at m/z 200 - 2000) were 20 and 120 ms, respectively. The ion target value for MS was set to 106 and for MS/MS to 105, and the intensity threshold was set to 8.3×10^2 .

For the whole proteome real-time tandem mass spectrometry was on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) with a 120-min acquisition time. Peptides were chromatographically resolved on an Ultimate 3000 RS-LC-nano System (Dionex), with an EASY-Spray PepMap RSLC, C18 stationary phase, 2 µm particle size, 100 Å pore size, 75 µm internal diameter x 500 mm length column (Thermo Fisher). The LC gradient conditions comprised of a flow rate of 0.3 µl/min and a gradient starting at 2% B (20% H₂O, 80% MeCN and 0.1% formic acid (FA)) and 98% A (0.1% FA, 100% H₂O) and increased to 27.5% B over 110 min followed by an increase to 40% B over 10 min, and finally increased to 90% B

over 1 minute. Peptides were analysed using a 375–1500 m/z scan range using quadrupole isolation at 120,000 resolution for an ion at 200 m/z. Tandem mass spectra were first collected using the ion trap and fragmented using 35% collisional induced dissociation (CID). A dynamic exclusion list was employed to prevent repeat sampling (repeat count of 2, repeat duration of 15 seconds, exclusion list size 100, and exclusion duration of 30 seconds).

2.2.5.2 Data analysis in MaxQuant

Data obtained from the mass-spectrometer are the intensity of ions (MS1) and their fragments (MS2). Sequences of 20-70% of peptides detected by the MS1 are identified with the help of fragments in MS2, the rest is excluded. The data were processed with MaxQuant version 1.5.3.8 (YnMyr Pull down), and 1.6.0.13 (Whole proteome). The peptides were identified from MS/MS spectra searched against the PlasmoDB-29_Plasmodium3D7_AnnotatedProtein database (15.12.2016) using the built-in Andromeda search engine. Because it is a database-based search engine, peptides containing unknown modifications will never be identified. All Thermo mass spectrometry .raw files were directly loaded into the MaxQuant software for quantification and identification. Protein intensity values were calculated based on the intensities of their corresponding peptides. Both LFQ (YnMyr Pull down) and TMT experiments (Whole proteome) analyses in MaxQuant were performed using the built-in algorithms. Cysteine carbamidomethylation was selected as a fixed modification, and methionine oxidation and N-terminal acetylation as variable modifications. For the pull down experiment, myristoylation was set as a variable modification using a modification composition of C(22) H(37) N(7) O(4) with a monoisotopic mass of 463.2907 on any N-terminus. Identification of modification sites using the YnMyr probe was also performed using the PEAKS proteomic software, which is discussed in section 2.2.5.3. For the modified peptide search two parameters were changed in MaxQuant: minimum score for modified peptide was reduced from 40 to 20, and the minimum peptide length was reduced to 6 from 7. This was to increase the chance of detecting the modified peptide. For enzyme digestion, trypsin was selected, which allows cleavage at the C-terminus of R and K residues and LysC which allows cleavage after K residues. Up to two missed cleavages were allowed. The false discovery rate (FDR) was set to 0.01 for peptides,

proteins and sites. Other parameters were used as pre-set in the software. 'Unique and razor peptides' mode was selected to allow identification and quantification of proteins in groups (razor peptides are uniquely assigned to protein groups and not to individual proteins), and all identifications were based on at least two unique peptides.

2.2.5.3 MS analysis for identification of the modified peptides

PEAKS is a *de novo* identification software assuming no prior knowledge of the database. Instead, their identification algorithm deduces the peptide sequence based on parent ion mass and fragmentation pattern. In PEAKS version 8.0, trypsin was set up as the digestion enzyme, precursor mass tolerance was set at 5.0 ppm, and fragment ion mass tolerance was set to 0.1 Da. An FDR threshold of 1% was used. Methionine oxidation and the aforementioned 463.2907 Da mass addition corresponding to the YnMyr-AzRB modification at protein N-terminal glycines were set as variable modifications. Carboxyamidomethylation of cysteines was set as fixed modification and PEAKS PTM was enabled to allow for additional modifications to be found. The peptide sequence matching was performed using the PlasmoDB-29_Plasmodium3D7_AnnotatedProtein database (15.12.2016).

2.2.5.4 Statistical Analysis on Perseus

The data were analysed using Perseus versions 1.5.6.0 for all experiments. Potential contaminants, reverse and proteins only identified by site were discarded before further analysis. The data were transformed using $\log_2(x)$ function (LFQ YnMyr Pull down) and $\log_{10}$ (TMT whole proteomic) before any further manipulation. LFQ data were normalized by subtracting the mean of each data column. TMT data were normalized by first subtracting the median from each data row, followed by subtracting the median of each column. The LFQ YnMyr pull down data and the TMT whole proteomic data were filtered to retain only those proteins that have at least three measurements across three replicates for every experimental condition and the data were imputed by replacing the missing values from a normal distribution with a width of 0.3 and a down shift of 1.8, which assumes missing values derive from low abundance proteins not reaching the detection limit. For multiple hypothesis-testing analysis, p-values were used for significance testing upon FDR

adjustment to account for the multiple testing problem. T-tests were performed using default Perseus settings, volcano plot representations have a visual cut-off line with parameters: $s_0 = 0.5 / 0.8$ for the LFQ pull down experiment and the whole proteomic respectively and an FDR cut off of 0.01.

Chapter 3. The *N*-myristoyltransferase inhibition phenotype is very variable and depends on inhibition conditions

As outlined in section 1.7.2, selective NMT inhibition leads to severe pleiotropic consequences for parasite development, presumably due to the malfunction of multiple downstream substrates. Since the expression of IMC proteins (GAP45, MyoA, MTIP and ISP3) is greatly reduced in NMT inhibited parasites, developmental arrests and cell death likely occurs before the synthesis of essential IMC proteins such as GAP45 at 38 h PI (Wright et al. 2014; Ridzuan et al. 2012). Therefore, the onset of the “pseudoschizont” phenotype might be initiated by an earlier different event preceding IMC malformation. Most of the NMT substrates identified in Wright *et al.*, are most abundant in schizogony and their expression commences with the onset of DNA synthesis at around 30 h PI (Wright et al. 2014). Treatment with NMT inhibitor for 45 h generates a severe phenotype, presumably with most NMT substrates not myristoylated and generating an accumulation of catastrophic events which lead to the death of the parasite at 34/36 h PI. To study the NMT inhibition phenotype in more detail and to determine individual NMT substrates responsible for lethality when not myristoylated, a shorter time window of inhibition was defined, which would still have a lethal phenotype. This chapter will present the data from selecting a shorter NMT inhibition period, sufficient to block development in late schizogony and subsequent invasion. In addition, it will present experiments to select a subset of six NMT substrates for an N-terminal G2A mutation screen, to determine the essentiality of myristoylation.

3.1 NMT inhibitor IMP-1002 blocks parasite development at 36 h PI leaving parasites with 4 to 5 nuclei and an equivalent number of genomes

In order to further understand the phenotype underlying NMT inhibition, a potent and selective *Plasmodium* spp. NMTi called IMP-1002 was used (Schlott et al. 2019). As described in section 1.7.1, IMP-1002 (Figure 1.7) was derived from a series of human NMTi (Mousnier et al. 2018). It is a close analogue of IMP-0917 (Figure 1.7) which

was discovered through a fragment reconstruction approach using hits from a screen against PvNMT and PfNMT (Bell et al. 2012). The inhibitor series around IMP-1002 was developed to overcome some of the limitations of the previously published PfNMT/PvNMT inhibitor, DDD85646 (compound 1a) (see Figure 1.7 and section 1.7.1) (Wright et al. 2014). This includes a 4-fold higher potency in killing parasites with an EC_{50} of 34 nM compared to 140 nM for DDD85646, as determined using the parasite growth assay (see section 2.2.1.10) (Schlott et al. 2019) (examined in MRes thesis) (Figure 3.1 A).

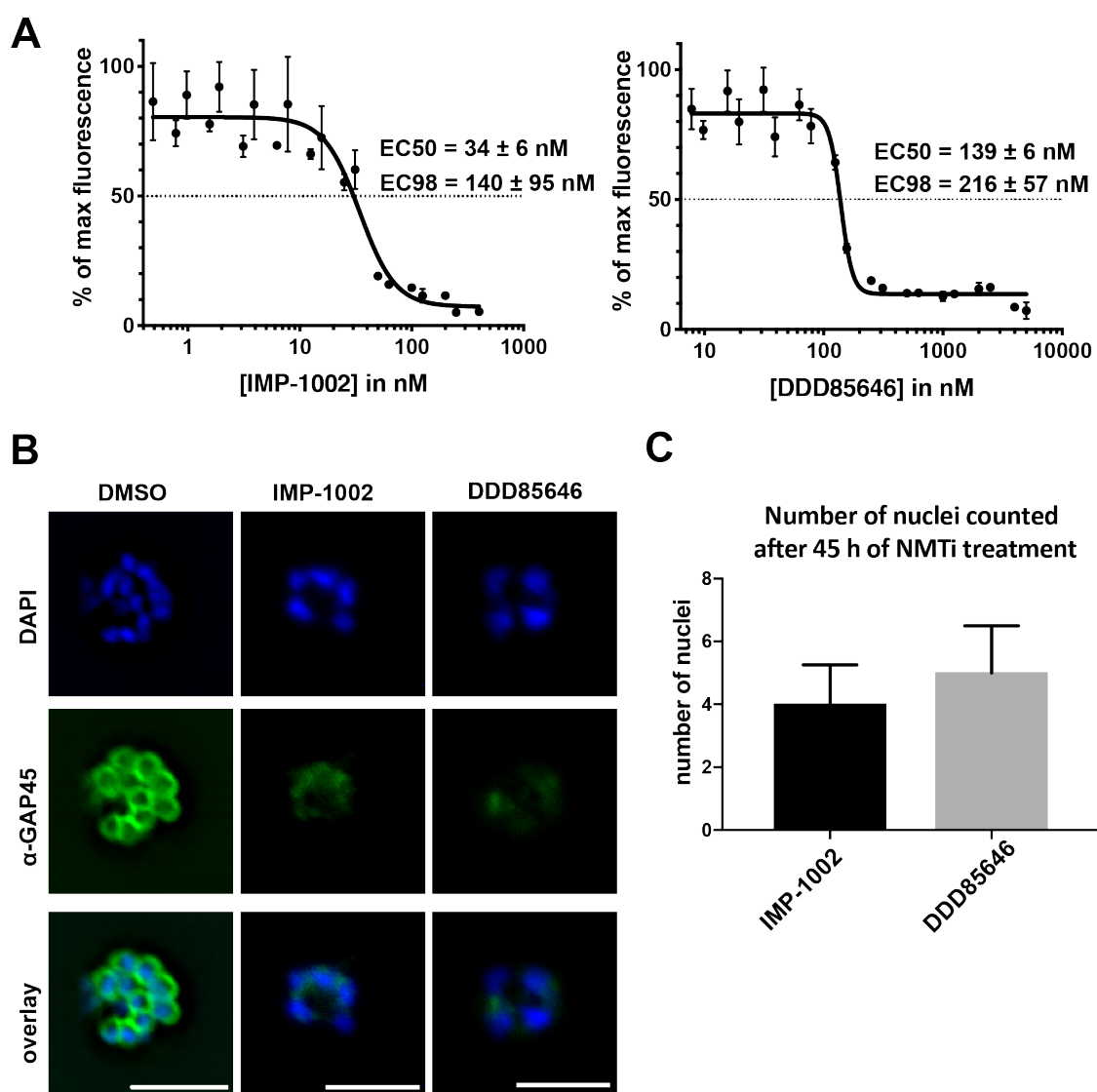


Figure 3.1 *P. falciparum* growth and development is inhibited by treatment with NMT inhibitors.

A. *P. falciparum* growth, measured by fluorescence of SYBR Green bound to genomic DNA, in the presence of increasing concentrations of NMT inhibitors. EC_{50} and EC_{98} values for compounds IMP-1002 and DDD85646 are indicated on the respective graphs (experiment was examined as part of MRes thesis). B. Indirect immunofluorescence

assay of parasites incubated from the ring stage with a control (DMSO) or four times the EC_{50} concentration of each NMT inhibitor (1 μ M DDD85646 and 140 nM IMP1002) and examined at 45 hours post invasion (h PI). Nuclei stained by DAPI (blue) and GAP45 (Inner membrane complex (IMC) indicator) localization in green. The number of nuclei in each cell and the abundance and location of GAP45 were affected by drug treatment (experiment was examined as part of the MRes thesis). All images were acquired with a Nikon PlanApo 100x/1.45 oil immersion objective. Scale bar = 5 μ m. C. Number of nuclei counted at 45 h PI for parasites treated with IMP-1002 or DDD85646 (n = 200 parasites per sample); error bars equal standard deviation (SD) of the median. Images adapted from Schlott *et al.*, 2019.

To determine whether inhibition with IMP-1002 and DDD85646 had the same phenotypic consequence, ring stage parasites were treated with the EC_{98} concentration of drug, as determined from the growth curve, and analysed by fluorescence microscopy using DAPI and GAP45 signals (Figure 3.1 B). Consistent with the results using NMTi DDD85646, treatment with IMP-1002 for 48 h reduced GAP45 signal and the number of nuclei was reduced from 20 nuclei in the DMSO control to 4 – 5 in the IMP-1002 treated sample (Figure 3.1 C) (Schlott *et al.* 2019) (examined in MRes thesis). The nuclei in these “pseudoschizonts” treated with IMP-1002, appear much larger in DAPI stained IFA images than those in DMSO treated fully segmented schizonts with 20+ nuclei (Figure 3.1 B). This led to the question whether the 4 – 5 nuclei identified in Figure 3.1C corresponded to an equivalent of 4 – 5 genomes, which would indicate that both DNA replication and nuclear division had stopped.

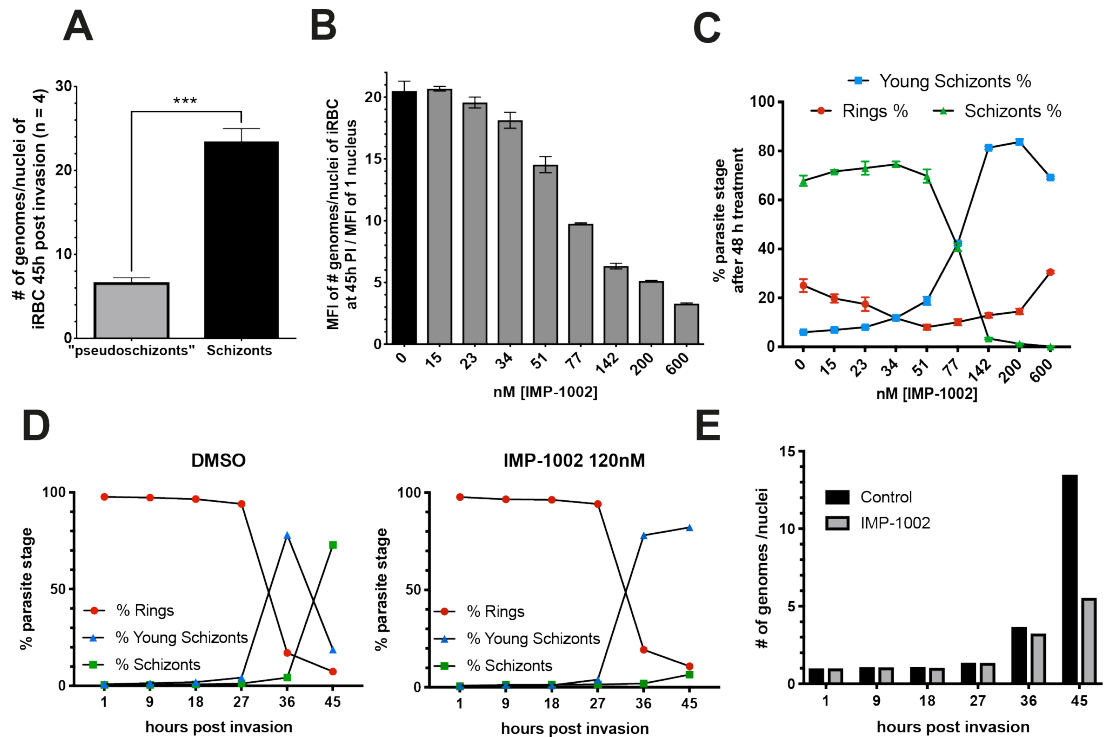


Figure 3.2 NMT inhibition results in an arrest of DNA replication and nuclear segregation.

The timing of the arrest changes with duration and concentration of inhibitor used. A. Graph showing significant difference in number of genomes (nuclei) determined through flow cytometry between “pseudoschizonts” and DMSO treated schizont-stage parasites ($n = 4$, $p = 0.0007$, unpaired t-test with Welch's correction). The median fluorescence intensity (MFI) of each sample was divided by the MFI of a sample of synchronized one-hour old rings containing one genome (nucleus) B. Dose dependent drop in number of genomes (nuclei) after 45 h of NMT inhibition with increasing concentration of the inhibitor. C. Dose dependent drop in mature schizont formation and increase in earlier schizonts after 45 h of NMT inhibitor treatment indicating delay in development. D. Parasite stages over the course of NMT inhibition. Schizont development starts at 27 h in both DMSO and NMTi treated samples, but at 36 h the development of mature schizonts is inhibited in the drug treated sample. E. Number of genomes (nuclei) over the course of NMT inhibition showing drop in schizont development at 36 h PI.

To investigate this, synchronised ring stage parasites were treated with 140 nM IMP-1002 and the number of genomes per infected erythrocyte was determined by flow cytometry using Hoechst as a fluorescent dye (see section 2.2.1.8). A synchronized one-hour old ring culture was fixed and the MFI was used as a reference point for cells with one nucleus. The MFI of pseudoschizonts in the presence of IMP-1002 and of schizonts in the presence of DMSO was determined and the value divided by the MFI of one nucleus (Figure 3.2 A). This showed that pseudoschizonts contain an amount of DNA corresponding to 4 – 5 nuclei and that this is significantly different from the amount in DMSO treated schizonts. This

“pseudoschizont” phenotype is irreversible and only observed for the time frame of drug treatment during the entire erythrocyte life cycle (Wright et al. 2014). As the concentration of IMP-1002 is increased at the ring stage, parasites stop progressively earlier in their development, as indicated by the reduced number of genomes after 45 h (Figure 3.2 B). The parasites shift from being fully mature schizonts to young “pseudoschizonts” after 45 h of drug treatment with increasing concentration of the drug (Figure 3.2 C). By following the maturation of parasites treated with the drug during the 1 – 45 h PI period in eleven-hour intervals, and using Hoechst staining, it is apparent that the block in schizont development occurs just after 36 h PI, with young schizonts still developing but not maturing into fully formed schizonts (Figure 3.2 D, E). This suggests that an increasing spectrum of substrates may become non-functional due to NMT inhibition as the NMTi concentration and time of exposure increase, leading to a variation in the phenotype as the conditions are changed.

In order to establish the on-target activity of IMP-1002 the EC_{50} was correlated with the concentration of drug necessary to cause a 50% decrease in myristoylation as measured by labelling through the myristic acid analogue YnMyr (YnC12) (see section 1.8 and 2.2.4.1). The tagging IC_{50} (TC_{50}) of IMP-1002 was determined based on the normalized fluorescence signal of the NMT substrate ARF1 ($n = 2$) after using the copper catalysed cycloaddition reaction to “click” the capture reagent containing a TAMRA fluorophore onto the alkyne of YnMyr as described in section 2.2.4.3 (Figure 3.3). As discussed in section 1.8 YnMyr is attached via an amide bond to the N-terminal glycine of NMT substrates, but also as part of a glycosylphosphatidylinositol moiety through a base-labile ester-linkage onto GPI-anchored proteins. This linkage can be removed through NaOH treatment to only visualise NMT substrates (section 2.2.4.4) (Figure 3.3 A). The IMP-1002 TC_{50} of 18.4 nM was determined using the ARF1 band intensity on gels both before and after NaOH treatment (Figure 3.3 B). The EC_{50}/TC_{50} ratio of 1.09 (Figure 3.3 B) provides a strong indication of the on-target activity of IMP-1002 since the same concentration is needed to kill 50% of the parasites in vitro and to reduce the signal of YnMyr labelling by 50%.

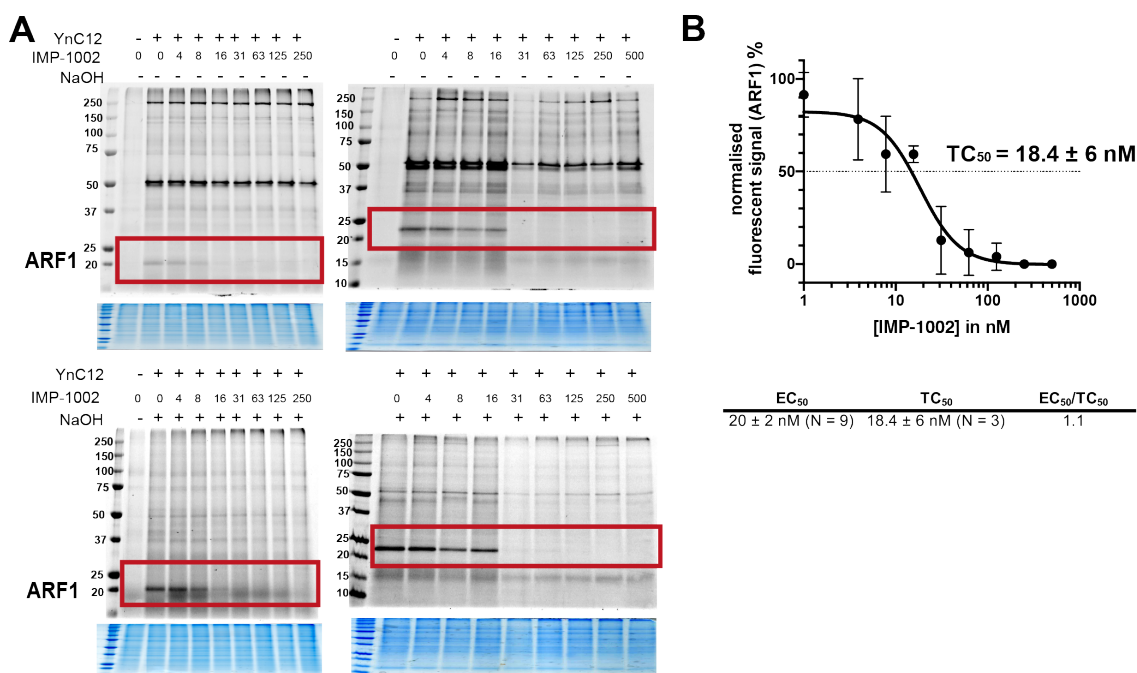


Figure 3.3 Tagging EC₅₀ (TC₅₀) calculation for IMP-1002.

A. SDS-PAGE gel of YnMyr labelled proteins prior (top) to and after (bottom) base (NaOH) treatment showing NMT substrates (e.g. ARF1 at ≈ 20 kDa) at different concentrations of IMP-1002. The gels (left and right) are from two independent biological replicates. Coomassie stain in blue shown below each gel. B. TC₅₀ calculation of both replicates using the intensity of the ARF1 band at ≈ 20 kDa (red box). The gel before NaOH treatment was also used for the intensity measurement (technical replicates). The signal was acquired through Fiji, normalized to the DMSO control and expressed as a percentage. Error bars indicate the standard deviation. Table below the graph indicate the ratio of EC₅₀/TC₅₀ which is an indication of the on-target activity of IMP-1002.

3.2 Inhibition of NMT for the last eleven hours of schizogony significantly decreases merozoite invasion rate and results in the significant drop in myristoylation of NMT substrates.

Variation in the length of NMT inhibition (using the EC₉₈ concentration of 140 nM, see Figure 3.4A) with progressively longer drug treatments at eleven-hour intervals, showed that only inhibition for >34 h is sufficient to generate the “pseudoschizont” phenotype (Figure 3.4A). With treatment starting later throughout the intraerythrocytic life cycle in eleven-hour intervals, parasites proceeded progressively further in their development (Figure 3.4B). Drug treatment only in ring and trophozoite stages (1-27 h PI) and for a short eleven-hour period was insufficient to arrest parasite development in early schizogony (Figure 3.4A, C). Since schizont development does not seem to be affected when parasites are treated with inhibitor

for the last eleven hours of the intraerythrocytic life cycle, a subsequent egress or invasion defect was investigated.

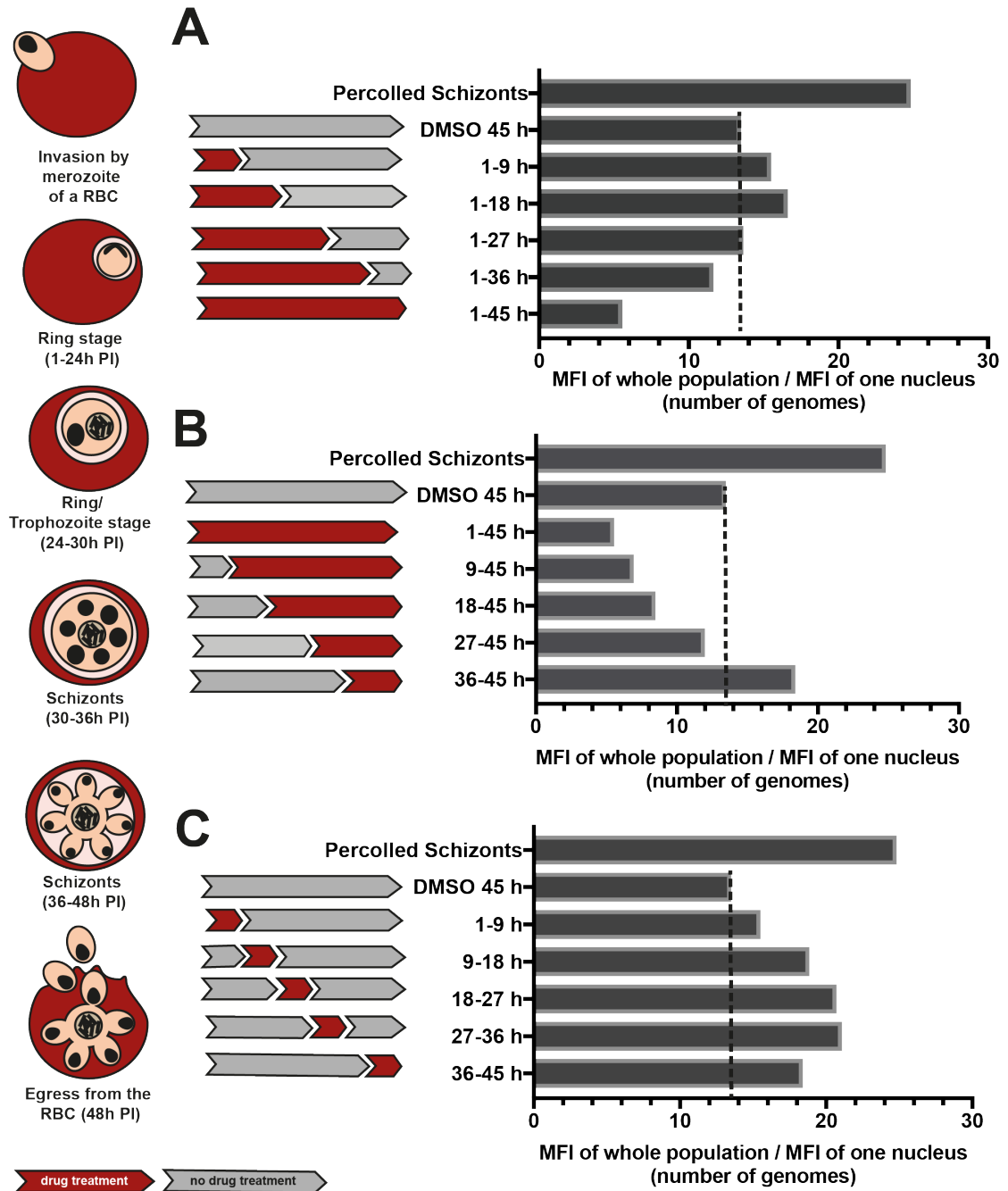


Figure 3.4 Variation in the length of NMT inhibition changes the phenotype dramatically.

A) Increase in the period of NMT inhibition reduces life cycle progression. B) Reduction in the period of NMT inhibition in nine-hour intervals allows parasites to progress further through their life cycle. C) Nine-hour periods of drug inhibition does not decrease the number of genomes (nuclei) in the parasite population after 45 hours post invasion (h PI). Dashed line marks the maturity of parasites in the control. Data were acquired using flow cytometry of Hoechst stained parasites. The mean fluorescence intensity (MFI) of

each sample was divided by the MFI of one-hour old ring-stages to determine the number of genomes (nuclei) per sample.

To investigate this, synchronized parasites were treated with either DMSO or 140 nM IMP-1002 from 34 – 45/48 h PI and parasites analysed using different methodologies (Figure 3.5A). For 3D7 parasites the intraerythrocytic cycle *in vitro* is 45 h instead of the 48 h that is expected *in vivo* (see section 1.3). The medium was exchanged to a drug free medium at the first sign of egress (around 45 h PI) and parasites were monitored by either microscopy of Giemsa-stained fixed parasites, flow cytometry, checked for invasiveness by an invasion assay, or harvested for mass spectrometry (see section 3.3), or an IFA (see section 3.5) (Figure 3.5A). It is important to note that starting parasitaemia needs to be adjusted for each type of assay separately (e.g. higher starting parasitaemia for Giemsa (4-7%), immunofluorescence and proteomics analysis and lower (>1%) for monitoring of parasites through flow cytometry, taking samples after egress).

Giemsa staining and microscopy indicated that a strong morphological phenotype was only apparent about 10 h after the exchange to a drug-free medium (55 h PI) with schizonts looking abnormal and very little sign of invasion (Figure 3.5B). In comparison, at 45 and 51 h PI drug-treated schizonts looked morphologically normal and comparable to those of the DMSO control (Figure 3.5B). At 62 h PI parasites that have escaped NMTi, develop into morphologically healthy looking trophozoites (Figure 3.5B). Flow cytometry analysis of samples stained with Hoechst dye (see section 2.2.1.8) demonstrated a significant drop in invasion in the cycle after drug treatment (Figure 3.5C) ($p > 0.0001$ for 51 h PI and 76 h PI samples, unpaired Student t-test with Welch's correction not assuming an equal SD for each time point individually, $n = 3$). To determine whether the decrease in invasion resulted from a decrease in egress or a defect in the invasion mechanism itself, an invasion assay was performed monitoring both ring and schizont populations separately (see section 2.2.1.9). Two samples (5 h after egress (53 h PI)) and at 24 h after egress (72 h PI)) as well as the schizonts starting population (at 45/48 h PI) were used to determine the parasitaemia and the invasion rate (Figure 3.5D left), as well as a percentage of rings and schizonts in each sample (Figure 3.5D right).

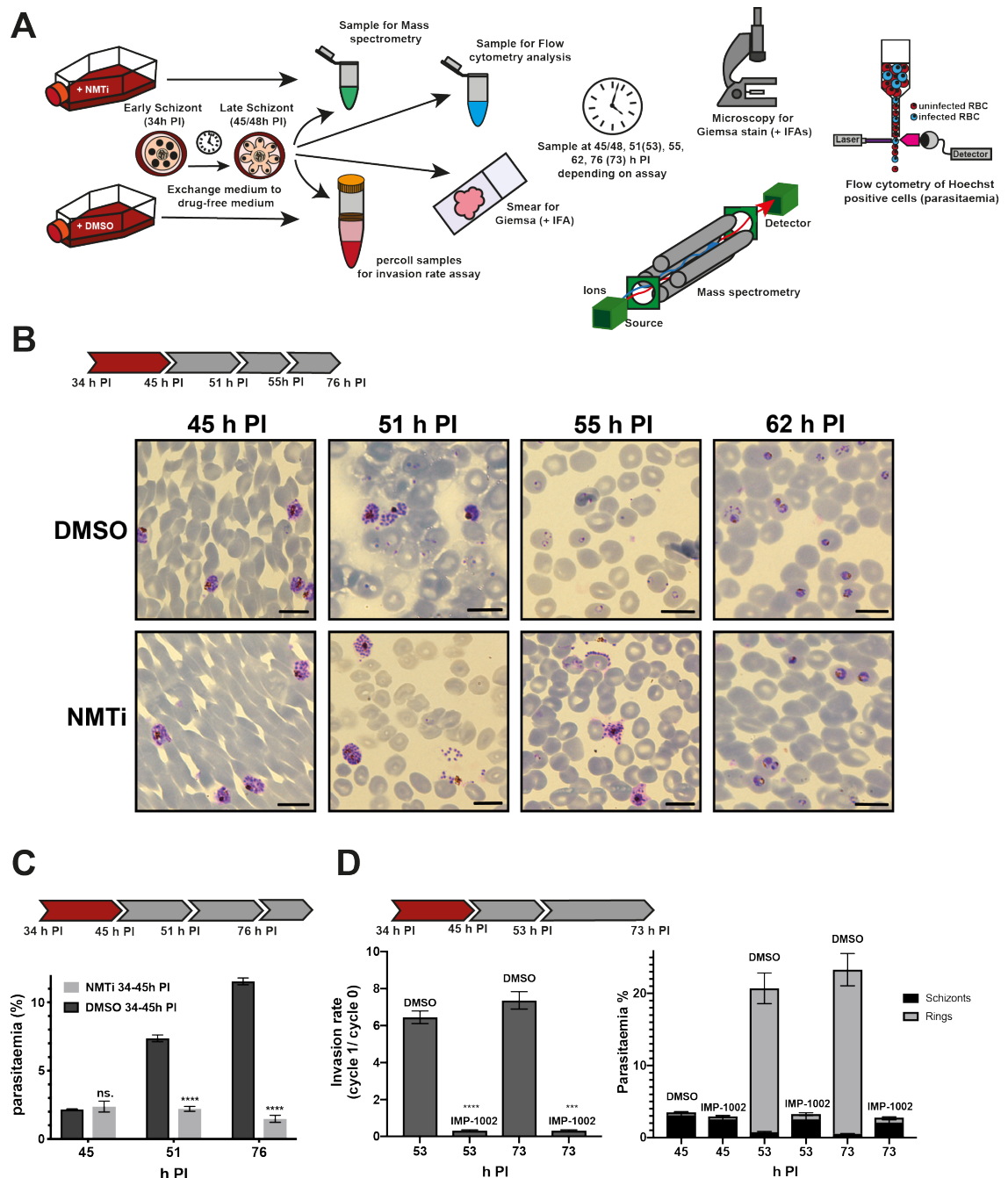


Figure 3.5 Inhibition of NMT leads to a block in development before egress.

Inhibition of NMT in the last eleven hours of the parasite's erythrocytic life cycle significantly reduces parasitaemia in the subsequent cycle through a block in processes leading on to egress. A. Parasites were treated with DMSO / NMTi from 34 to 45/48 hours post invasion (h PI). At the first sign of egress in the control, the medium was exchanged to drug free medium. Subsequently in two hours intervals, samples were collected for microscopy (Giemsa-stained and immunofluorescence) and flow cytometry. For a separate experiment, parasites were harvested for proteomics at 45 h PI and for a third experiment parasites were percoll-purified at 45 h PI and an equal number of schizonts were mixed with fresh RBC to start an invasion assay taking samples at 53 and 73 h PI in the second cycle. B. Giemsa stain of the morphology of parasites after eleven-hour drug treatment. While there is no morphological difference visible between

DMSO control and IMP-1002 treated samples at 45 and 51 h PI, after 55 h PI abnormalities in schizont morphology become apparent. Parasites escaping drug treatment develop normally into trophozoites. Scale bar = 10µm. C. Parasitaemia was significantly reduced in NMTi treated sample ($p > 0.0001$ for 51 h PI and 76 h PI, unpaired student t-test with Welch's correction not assuming an equal SD for each time point individually, $n = 3$). D. Invasion assay showing invasion rate at 53 and 73 h PI (parasitaemia of cycle 1 / cycle 0) (left) and percentage of schizonts and rings (right). The invasion rate drops significantly ($p > 0.0001$ for 53 h PI and $p > 0.0003$ for 73 h PI, unpaired student t-test with Welch's correction not assuming an equal SD, $n = 3$). While the schizont population stays the same between 45/48 – 72 h PI in the IMP-1002 inhibitor treated samples, the schizont population drops in the DMSO control and the ring population increases. In each subfigure the time of drug treatment is indicated by a red arrow and the time points of sample collection by a grey arrow.

The invasion rate drops significantly ($p > 0.0001$ for 53 h PI and $p > 0.0003$ for 73 h PI, unpaired student t-test with Welch's correction not assuming an equal SD, $n = 3$) supplementing the drop in overall parasitaemia observed earlier (Figure 3.5C). While the schizont population decreases between 48 – 72 h PI in the DMSO control indicating egress and resulting in an increase of the ring population, the schizont population of the IMP-1002 treated samples remained at the same level between 48 – 72 h PI with little rise in the percentage of ring stage parasites (Figure 3.5D right). This indicates an egress phenotype since there was no increase in ring stage parasites in combination with a constant schizont stage population. This result complements those from the Giemsa images, indicating that merozoites fail to egress leading to a decrease in parasitaemia. All assays indicate that a small percentage of parasites escape NMT inhibition. Unpublished data has shown that NMTi show a killing lag phase of 24 h with an asynchronous culture of parasites, similar to the killing rate of pyrimethamine (see PhD thesis of Andrew Bell (Bell 2016) who synthesized the inhibitor series around IMP-1002 (see section 1.7.1)). This explains why an eleven-hour window of inhibition is insufficient to reduce the parasitaemia to 0% and why some parasites escape the drug inhibition.

3.3 IMP-1002 inhibits myristoylation *in vitro*.

The inhibition of NMT in the last eleven hours of the erythrocytic life cycle leads to a significant blockade in the events leading to egress and a resulting decrease in parasitaemia in the subsequent cycle. In order to understand the biology underlying the consequences of NMT inhibition, the effect of NMT inhibition on myristoylated

substrates in the last eleven hours of the cycle was analysed. This was done using a quantitative and comparative chemical proteomic approach utilizing the myristic acid analogue YnMyr (see section 2.2.4.1). In comparison to what was used for the in-gel fluorescence (see section 3.1), a capture reagent, AzRB that contains a trypsin cleavable linker as well as a biotin tag, was used to pull down labelled proteins that have incorporated YnMyr, with Neutravidin resin (see section 2.2.4.3 and 2.2.4.8). The labelling of GPI anchored proteins through YnMyr served as an internal control since this incorporation should not respond to inhibition of NMT and the intensity should be the same between equivalent treated and untreated samples.

After Neutravidin-precipitation, samples were reduced, alkylated and digested with trypsin (see section 2.2.4.8) before peptide analysis on a Thermo Q-Exactive mass-spectrometer (see section 2.2.5.1). For this experiment, label free quantification (LFQ) was used as the quantification method (see section 1.8.2) to determine the difference in relative amount of individual proteins successfully pulled down from DMSO- and IMP-1002-treated samples based on the peak ion intensity of peptides in the two preparations. Overall, there were 1107 *P. falciparum* proteins identified in the two samples. However, 447 proteins were not used for the analysis based on four criteria: because there was no associated intensity value present in all three biological replicates, they were classified as potential contaminants, they were only identified by a peptide carrying a modified residue ("only identified by site"), and they were identified only through the reverse sequence in the decoy database ("reverse"). Furthermore, there were 51 human proteins identified, which were also excluded from the analysis. The MS peak intensity for 609 proteins was used and normalized by subtracting the mean intensity of all proteins (see section 2.2.5.4). A t-test identified sixteen NMT substrates that show a significant decrease in myristoylation in the presence of the NMTi (Figure 3.6) (adjusted p-values using FDR of 0.01 and within group variance $S_0 = 0.5$, $N = 3$). All sixteen proteins show a ≥ 2.7 -fold decrease ($\log_2$ fold change of -1.4) and a p-value $\leq$ than 0.01 ($-\log_{10}(2)$) (Figure 3.6). Fourteen of those proteins are known NMT substrates marked in pink, and one protein is a predicted NMT substrate (Wright 2013) with no known function (Figure 3.6). This leaves a putative kinase that contains an N-terminal glycine (marked with a diamond shape indicating the presence of an N-terminal glycine) but the modified peptide has not been identified to date and the protein was not identified by any bioinformatic prediction software (Wright et al. 2014). Nevertheless, the significant response to

NMT inhibition as well as the presence of the N-terminal glycine indicates that this protein could be an NMT substrate.

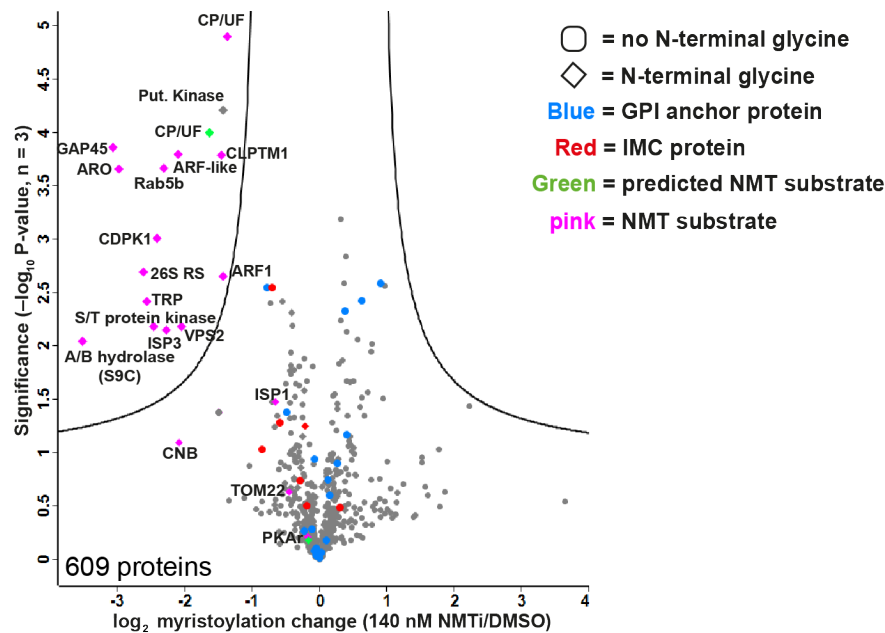


Figure 3.6 NMT inhibition changes myristoylation levels.

Proteomic label free quantification (LFQ) analysis of the inhibition of protein myristoylation in *P. falciparum* parasites treated from 34 to 45/48 h PI after ligation to AzRB and enrichment on NeutrAvidin coated agarose beads. A two-sample t-test (permutation-based false discovery rate [FDR], 250 permutations (number of randomizations), FDR 0.01, $S_0 = 0.5$ (within groups variance), $n = 3$) revealed significant changes in LFQ between 140 nM IMP-1002-treated and the control (DMSO) samples for the abundance of myristoylated proteins. Line on graph indicates t-test significance cut off. Legend at top right of graph explains symbols and colour coding of individual proteins in the plot.

GPI anchor proteins marked in blue as well proteins associated in a complex with known NMT substrates such as IMC proteins marked in red, do not respond to NMT inhibition although they have been pulled down through YnMyr labelling or by interacting with NMT substrate proteins (Figure 3.6). The proteomic data set was also used to identify the peptide carrying the site of modification using PEAKS (see section 2.2.5.3) (Table 7.4 in appendix). This serves as an unambiguous means to verify a protein as an NMT substrate. Compared to the NMT substrates identified in Wright *et al.*, two additional modified peptides were found from: vacuolar protein sorting-associated protein 2 (VSP2) and acylated pleckstrin-homology domain-containing protein (APH). Additionally, the modified peptides of two predicted NMT substrates (metal-dependent protein phosphatase 6 (PPM6) and multidrug

resistance protein 1 (MDR1)) were found, which have not been previously classified experimentally as NMT substrates (Wright et al. 2014) (Table 7.4 in appendix).

3.4 NMT inhibition changes protein abundance of several NMT substrates

The experiment described above was designed only to identify changes in enrichment of YnMyr labelled proteins. It is, however, possible that the overall protein abundance is affected as a downstream consequence of NMT inhibition. Therefore, the whole proteome of parasites treated from 34-48 h PI with and without 140 nM IMP-1002 in biological triplicates was also subjected to a mass spectrometry analysis to identify changes in protein abundance as a result of NMT inhibition. iRBC pellets were subjected to saponin lysis and protein extracted (see section 2.2.4.2), and then prepared for LCMS/MS (see section 2.2.4.9). To reduce sample complexity and increase coverage of the whole proteome, samples were divided into fractions before LCMS/MS analysis (Figure 3.7) (see section 2.2.4.10). However, this increased the sample number, and resulted in the decision to use TMT (tandem mass tag) labelling of the biological triplicates of the two conditions with six different isobaric tags (Figure 3.7) (see section 1.8.2). Allowing multiplexing of samples is only one advantage of TMT labelling. It also provides additional robustness because measurement of different samples is made within the same MS2 spectra by selecting common precursor ions corresponding to the same peptide in all samples, rather than at the MS1 level used for LFQ. The identified peptides were searched in MaxQuant against the PlasmoDB and the uniprot human databases (see section 2.2.5.2) which identified 2930 proteins. Only 2731 proteins were selected for further analysis after filtering (see section 2.2.5.4) and a further 247 human proteins were excluded. A t-test using an FDR cut-off of 0.01 and a within-group variance (S_0) of 0.8 resulted in identification of 62 proteins significantly reduced in abundance due to NMT inhibition (Figure 3.8 and Table 7.5 with larger table provided in excel file). Significant proteins showed up to 530-fold decrease in signal. Six of the significant proteins are known NMT substrates (ARO, GAP45, CDPK1, tetratricopeptide-repeat protein (TRP), S9C and ISP3) (Figure 3.8). Those substrates decreased 4-fold to 16-fold compared to the DMSO sample. TRP was not discussed previously in section 1.5.

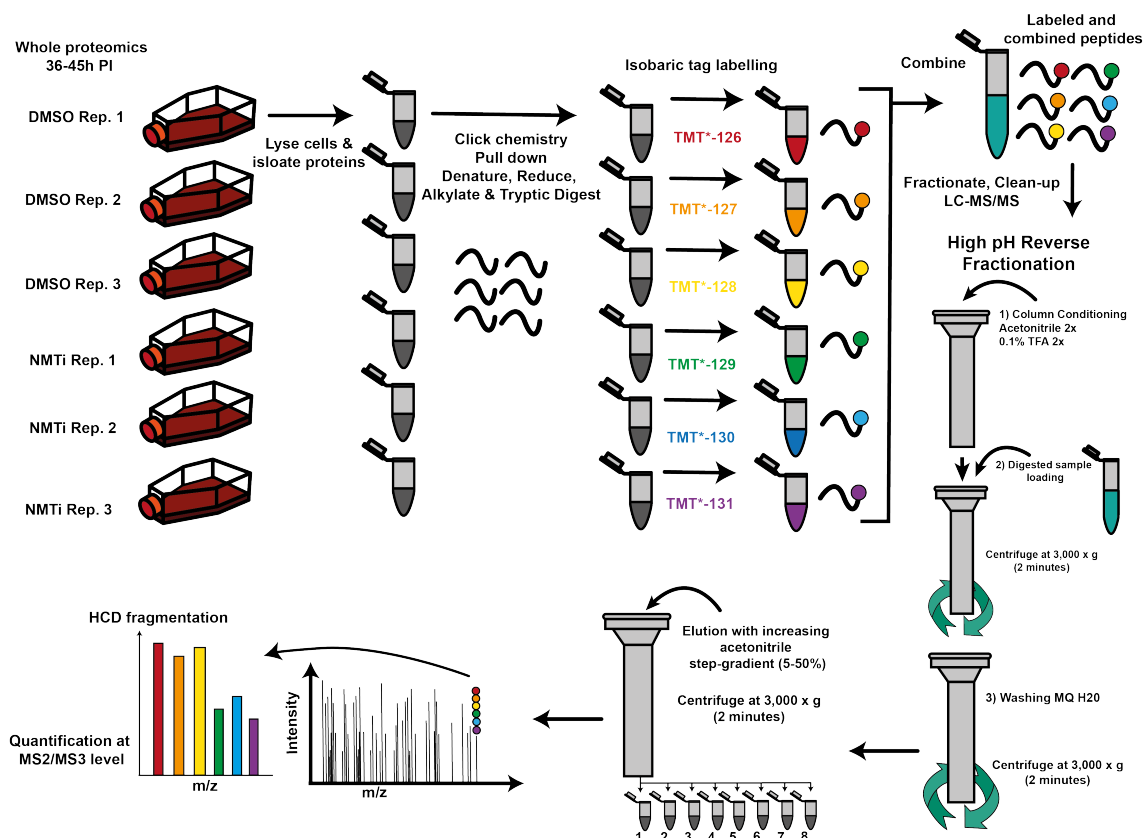


Figure 3.7 Tandem mass tag (TMT) labelling workflow for quantitative whole proteomics.

Parasites are treated with the NMTi and DMSO control from 36-45 h PI. After protein extraction, click chemistry, pull down, and on-bead digestion with trypsin, peptides in the different samples were labelled with a specific TMT tag, which are attached to primary amine groups and allow relative quantification of the samples. Samples were combined and subjected to high pH reverse fractionation with a gradient stepwise elution from 5-50% acetonitrile to decrease the complexity of the sample and therefore increase the coverage of the proteome. Samples were analysed by liquid chromatography tandem-mass spectrometry (LC-MS/MS). TMT reagents are isobaric, meaning they have the same mass and structure, and therefore co-elute during LC-separation. However, each isobaric reagent contains a different number of heavy isotopes in the mass reporter region, which results in a unique reporter mass during tandem MS/MS for sample identification and relative quantitation.

This protein exists in two different paralogues (PF3D7_0601600 and PF3D7_0631000) which differ by one amino acid deletion and one substitution. One isoform (PF3D7_0601600) was classified as dispensable in the recent *P. falciparum* mutagenesis screen (Zhang et al. 2018) while the other was classified as essential (PF3D7_0631000). Nothing is known about their cellular location or function. Through GO term analysis on PlasmoDB (new search; genes; annotation, curation and identifiers; gene IDs; search; analyse results; go term analysis) 24 proteins were identified that have functions related to DNA synthesis such as 7 histone proteins,

12 are exported/secreted proteins, 3 proteins are associated with the IMC, one chaperon protein, one protein involved in transport of vesicles during budding, one SUB1-like peptidase and 14 are conserved proteins which have no reported function (Figure 3.9) (see Table 7.5 in appendix, for larger table please see provided excel file).

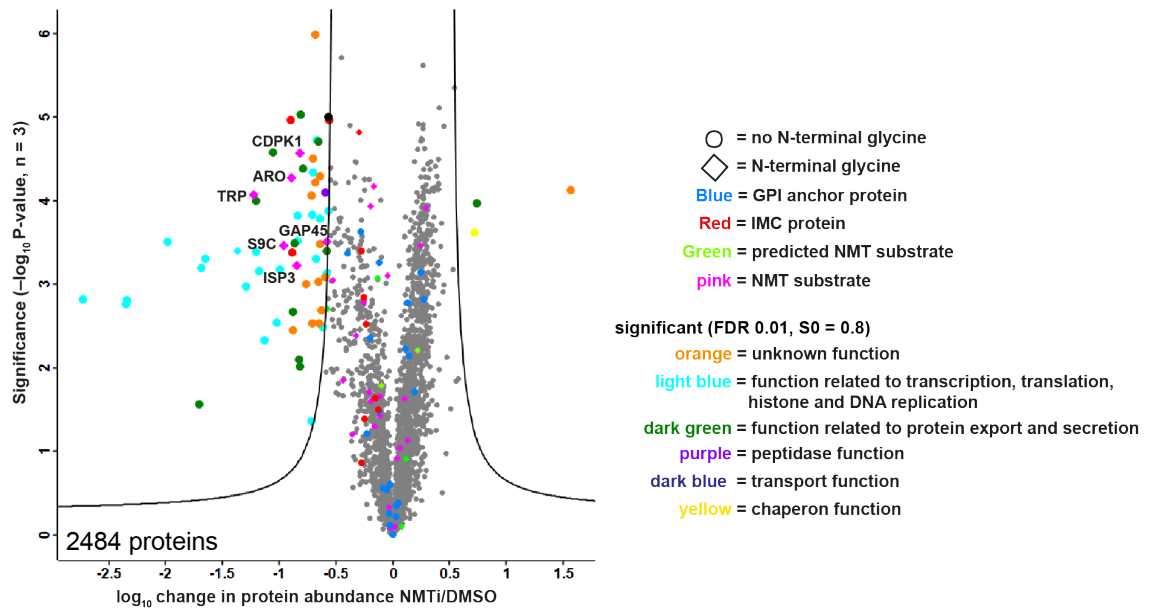


Figure 3.8 NMT inhibition causes changes in myristoylation levels leading to a loss in overall protein abundance.

Quantitative whole proteomic analysis using tandem mass tag (TMT) labelling of protein abundance in samples treated either with DMSO or 140 nM IMP-1002 with subsequent saponin lysis. A two-sample t-test (permutation-based FDR, 250 permutations, FDR 0.01, $S_0 = 0.8$ (within groups variance), $n = 3$) revealed significant changes in TMT labelling between 140 nM IMP-1002 and the control (DMSO) in overall protein abundance. The legend to the right of the graph explains the symbols and colour coding for individual proteins on the plot.

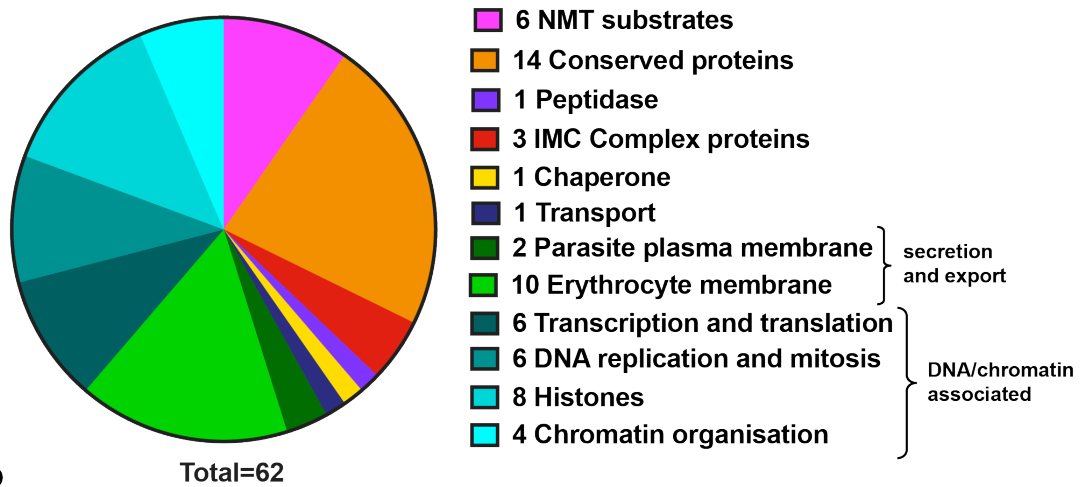
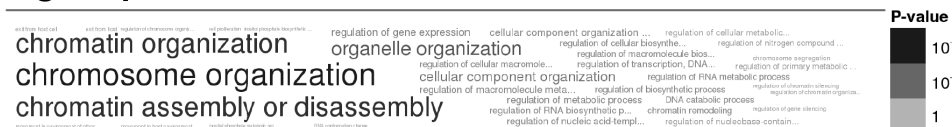
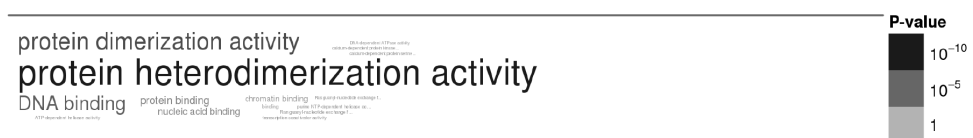
A**B****Biological processes****Cellular component****Molecular function**

Figure 3.9 Gene ontology (GO) term analysis of NMT inhibition whole proteomics experiment.

Analysis for proteins significantly reduced in abundance following IMP-1002 treatment from 34 to 48 h PI. A. Pie chart of the 62 proteins significantly reduced in abundance by IMP-1002 treatment with their associated protein group based on the GO term analysis from PlasmoDB. B. GO term enrichment in biological processes, cellular component and molecular function of the 62 significantly reduced proteins upon IMP-1002 treatment.

3.5 Dual acetylation ensures membrane anchoring while NMT inhibition leads to cytoplasmic localisation

To further analyse the subcellular morphology of the NMTi phenotype after treatment with IMP-1002 for the last eleven hours of the erythrocyte life cycle, samples were

analysed using an indirect IFA and specific antibodies (see section 2.2.3.1) (Figure 3.10). IMC proteins GAP45 (NMT substrate), MyoA and GAP50 were all slightly reduced in abundance but still localised towards the periphery of merozoites, indicating a morphological normal development and formation of the IMC (Figure 3.10A, B). MSP1 is located on the outside of the parasite plasma membrane in developing merozoites, and its localisation did not change upon IMP-1002 treatment when comparing schizonts of the same segmentation stage (Figure 3.10B). The micronemal protein EBA-175 is also not affected by IMP-1002 treatment (Figure 3.10C). The localisation of rhoptry proteins ARO (an NMT substrate localised at both rhoptry bulb and neck by analogy with its TgARO orthologue (C. Mueller et al. 2013)) (see section 1.5.2) and RON4 (localized at the rhoptry neck (Roger et al. 1988)) changed from a discrete to a very diffuse localisation in the cytoplasm as well as at the periphery of the merozoites, which resembled MSP1 staining (Figure 3.10D). Depending on the maturity of the schizont, the extent of mislocalisation after treatment had different degrees of severity, with some schizonts having ARO and RON4 partially localised at the rhoptries and some with a distribution resembling MSP1 staining (Figure 3.10D(5)). Furthermore, younger schizonts in the DMSO treated control also show partial lack of rhoptry localisation for both ARO and RON4 (Figure 3.10D(1)). As the schizonts mature, it seems that ARO localizes first to the rhoptries (Figure 3.10D(1)), while RON4 still has a MSP1-like distribution (Figure 3.10D(3,4)). Only in fully segmented schizonts are both RON4 and ARO localised at the rhoptries (Figure 3.10D(2)). However, in the IMP-1002 treated sample there was no signs of rhoptry localisation of the two proteins.

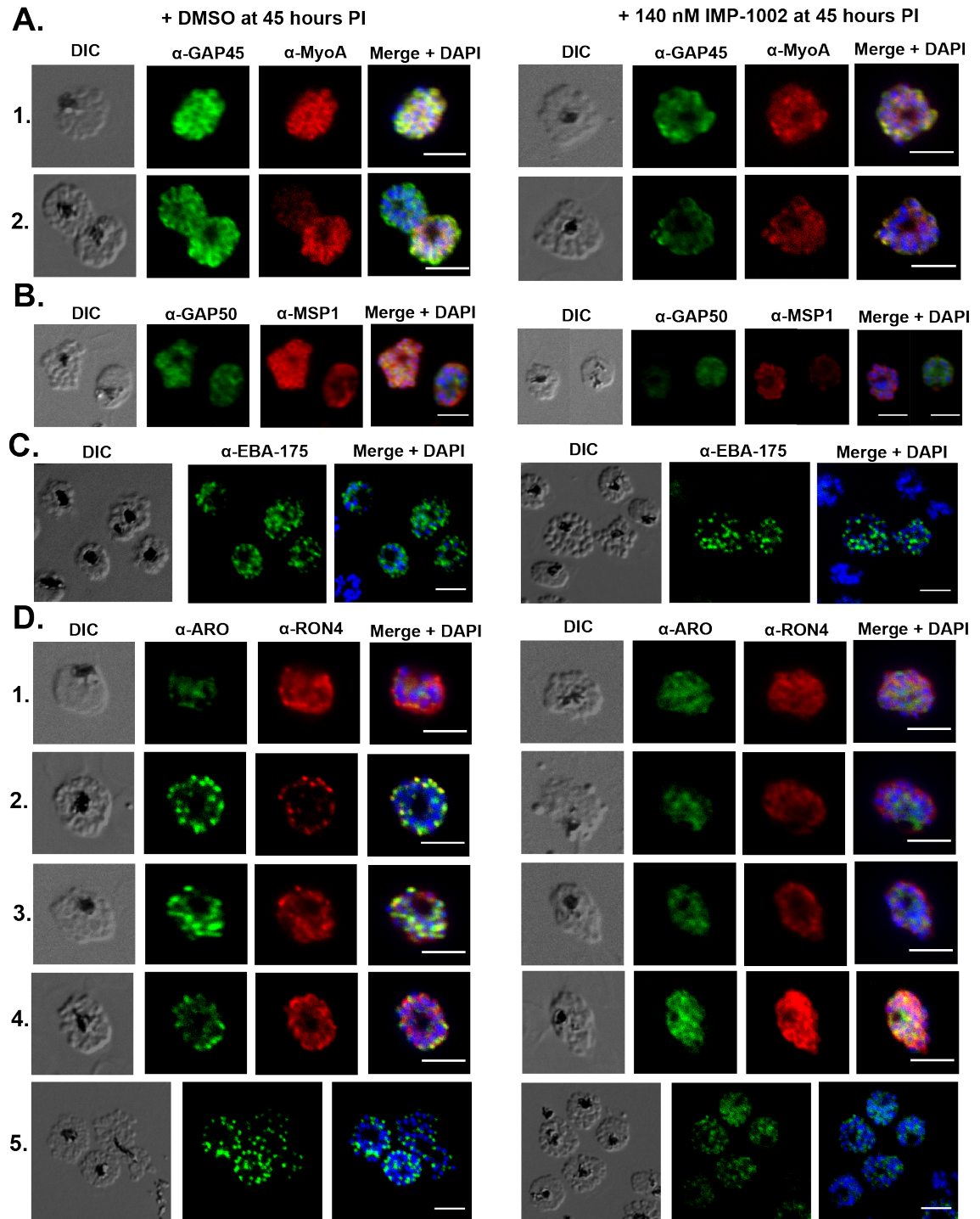


Figure 3.10 NMT inhibitor (IMP-1002) treatment from 34 - 48 h PI changes the localisation of NMT substrate ARO and rhoptry protein RON4.

Images shown an indirect immunofluorescence assay with fixed parasites. A) GAP45 and MyoA localisation at the inner membrane complex (IMC) was largely unchanged following NMT inhibition from 34 to 48 h PI. Comparing parasites with the same segmentation, the GAP45 signal is slightly reduced but localised at the periphery of merozoites. B) Both MSP1 and GAP50 signals were similar in IMP-1002 and DMSO treated parasites. C) The location of the micronemal protein EBA-175 was not affected by IMP-1002 treatment. D) Five images of DMSO and IMP-1002 treated schizonts

showing ARO and RON4 localisation at the rhoptries. In the inhibitor treated sample both RON4 and ARO localisation changed from the rhoptries to the periphery of merozoites. All images were acquired with a Nikon PlanApo 100x/1.45 oil immersion objective. Scale bar = 5 μ m.

Considering the significant reduction of ARO in the whole proteomic analysis of the NMTi treated sample (see section 3.4), it was surprising not to see a change in abundance of ARO upon IMP-1002 treatment using IFA. The main difference between the samples used for IFA and for whole proteomics was the processing step prior to analysis. While IFA used intact parasites fixed on microscope slides using PFA, the whole proteomic analysis used saponin-lysed parasites which have been separated from the host's RBC membrane. Saponin was expected to only disrupt the RBC and PV membrane leaving the parasite membrane intact (Burghaus & Lingelbach 2001). However, to establish whether this treatment led to the loss of some soluble proteins, the whole proteomic analysis was therefore repeated with intact percoll-purified parasites (Figure 3.11A right). Compared to the samples treated with saponin (Figure 3.11 A left, same as Figure 3.8, shown twice for direct comparison of +/- saponin), there was a larger contamination with host cell proteins (354 human proteins were detected in intact schizonts and 247 human proteins were in saponin-lysed samples). In addition, the sample was increasingly complex due to haemoglobin contamination which reduced the stage tip clean-up efficiency on reverse phase C18 SDB-XC (see section 2.2.4.9) and therefore might have reduced the coverage, making changes between samples more difficult to detect. Three NMT substrates (TRP, ISP3 and S9C) significantly decreased in abundance in extracts of intact schizonts, as determined through t-test analysis (permutation-based FDR, 250 permutations, FDR 0.01, $S_0 = 0.8$ (within groups variance), $n = 3$). One NMT substrate (an ARF-like protein) and a H3 histone protein showed a slight increase in abundance (Figure 3.11A right).

Western blot analysis of lysate before and after saponin treatment as well as before and after IMP-1002 treatment, was used to confirm the reduction in protein abundance of ARO, CDPK1 and GAP45. All three substrates showed a decrease in abundance on western blot of schizonts following IMP-1002 treatment, which was more pronounced in extracts of saponin lysed parasites (Figure 3.11B). ARF1, which showed no significant reduction in the whole proteomic analysis also showed no

reduction on the western blot; neither did HSP70 and BIP, both of which are not substrates of NMT.

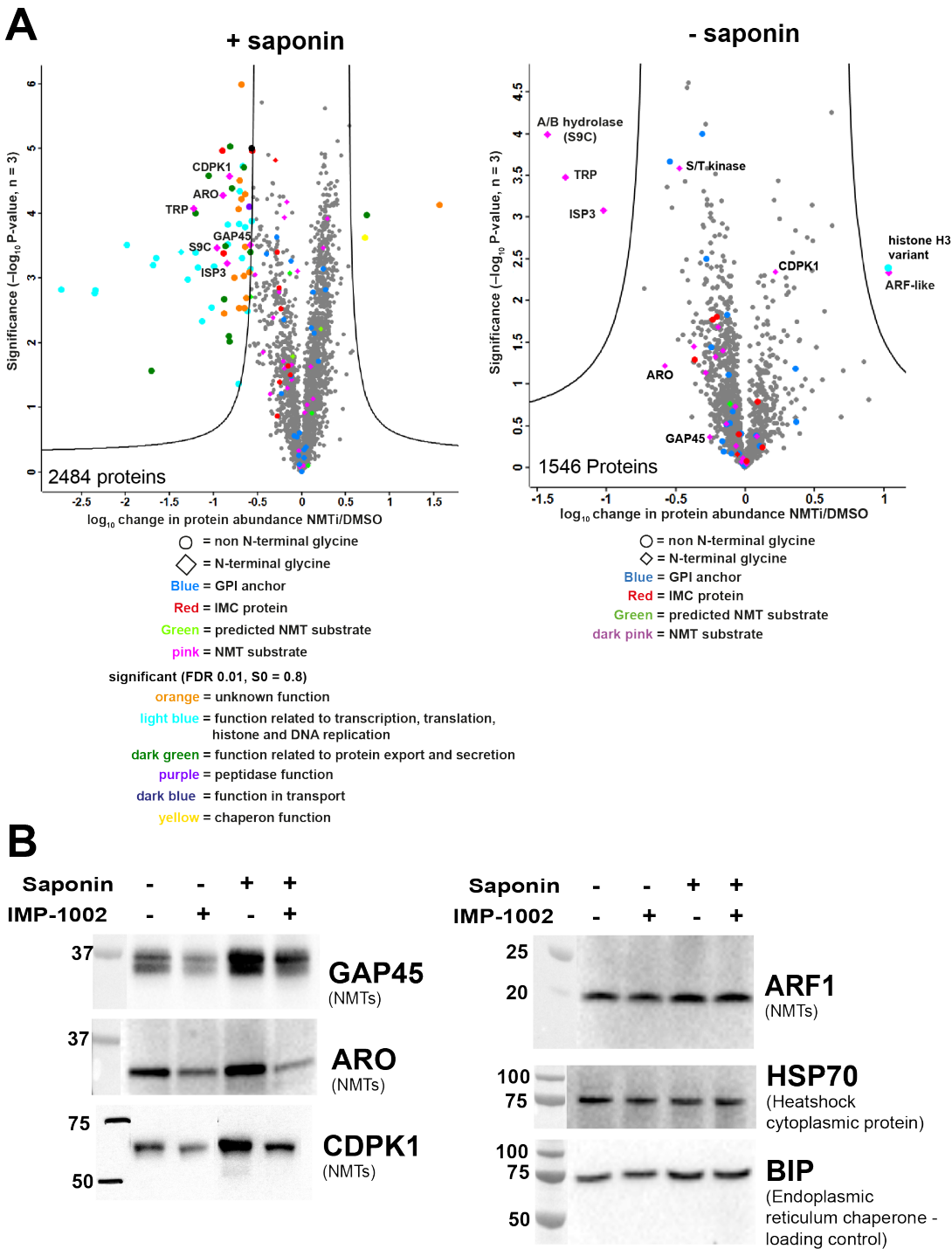


Figure 3.11 Protein abundance of selected NMT substrates changes in both intact and saponin-lysed parasites after IMP-1002 treatment.

A. Quantitative whole proteomic analysis using tandem mass tag (TMT) labelling of peptides derived from saponin lysed schizonts (left – same as Figure 3.8) and intact schizonts (right). Graph shows protein abundance in DMSO and IMP-1002 (140 nM)

treated samples. A two-sample t-test (permutation-based FDR, 250 permutations, FDR 0.01, S_0 (within groups variance) = 0.8, $n = 3$) was used for both experiments and revealed significant differences in overall protein abundance in the TMT-labelled samples treated with 140 nM IMP-1002 and the control (DMSO). The legend below the graphs explains the symbols and colour coding of individual proteins in the plot. Contaminant human proteins have been excluded from the analysis. B. Western Blot showing NMT substrates from parasites treated or untreated with NMT inhibitor from 34 to 45 h PI (10 μ g of lysate). While ARF1 was not reduced in abundance, GAP45, CDPK1, and ARO decreased in both intact and saponin lysed parasite fractions from cultures that had been IMP-1002 treated. BIP, which is an endoplasmic reticulum chaperone protein, is not an NMT substrate, showed no change in abundance upon NMTi treatment, and was used as a loading control. HSP70 is a cytoplasmic protein that is also not an NMT substrate and its abundance was not affected by NMT inhibition.

To determine whether NMT substrates present in samples treated with or without inhibitor have different membrane associations, schizonts were submitted to subcellular fractionation using hypertonic and high salt buffer to release cytosolic proteins, followed by high pH carbonate extraction. This fractionation will solubilise peripheral membrane proteins (carbonate soluble) away from integral membrane proteins (carbonate insoluble), since only tightly associated membrane proteins such as integral membrane proteins would fail to solubilise by a high pH carbonate buffer (Ridzuan et al. 2012). While CDPK1 and ARO (both containing N-terminal myristoylation and palmitoylation) were identified as integral membrane proteins in the carbonate insoluble fraction from the DMSO control parasites, upon NMTi treatment both proteins either partially (in the case of CDPK1) or completely (in the case of ARO) were present in the hypertonic soluble fraction (Figure 3.12). GAP45 localisation did not change due to NMT inhibition, and it remains in the carbonate insoluble fraction. Controls for cytoplasmic proteins (HSP70, a heat shock protein and Sera5, a soluble protein of the PV), a peripheral membrane protein (MSP7) and an IMC glideosome complex member (MTIP) were present in the expected fractions.

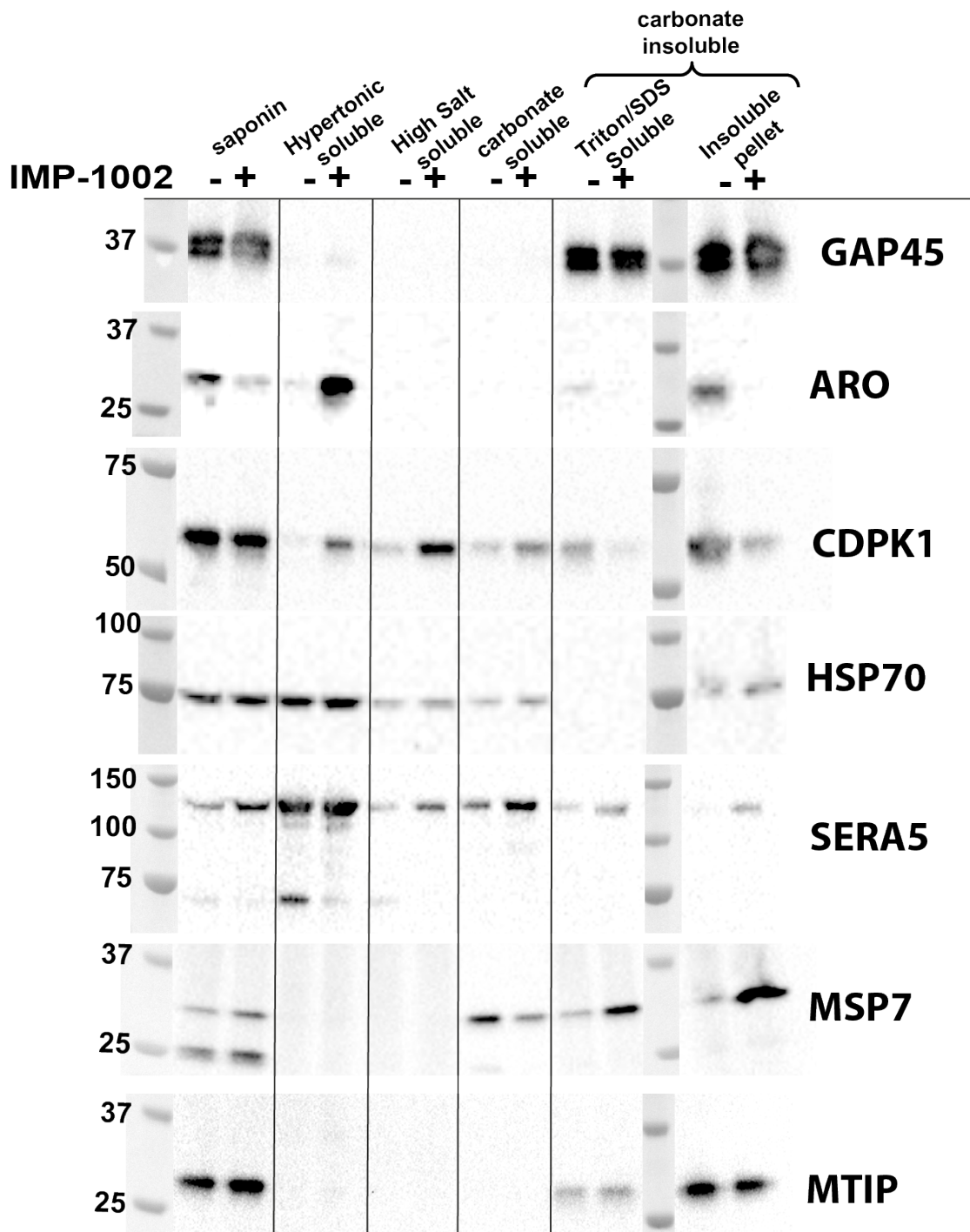


Figure 3.12 Schizont lysate fractionation after treatment from 34 - 48 h PI with 140 nM IMP-1002 and DMSO control.

Subcellular fractionation of percoll-purified infected intact RBC at 48 h PI, was performed sequentially using hypertonic lysis buffer, high salt buffer, sodium carbonate buffer and 1% Triton X100, 0.1% SDS buffer. All fractions were analysed by western blot using antibodies to GAP45, ARO, CDPK1, HSP70, SERA5, MSP7 and MTIP. Comparing IMP-1002 treated and untreated parasites, GAP45 remained in the carbonate-insoluble pellet (Triton/SDS and insoluble pellet), while ARO and CDPK1 partitioned into the hypertonic and high salt lysates in NMTi treated parasites, comparable to the cytoplasmic protein HSP70. Both ARO and CDPK1 remained in the carbonate insoluble fraction in DMSO

treated parasites. Controls for soluble (HSP70 and SERA5), peripheral membrane (MSP7) and carbonate insoluble IMC complex protein (MTIP) were present in their respective fractions.

3.6 A G2A/G2G CRSPR/Cas9 viability screen allows the selection of NMT substrates for further analysis

The sixteen NMT substrates showing significant changes in myristoylation upon NMT inhibition (Figure 3.6) were analysed based on their function, location within the parasite, known additional modification at N- or C-terminus through for example palmitoylation, and whether or not previous mutagenesis screens have classified them as essential (mainly through the *P. falciparum* piggyBac mutagenesis screen (Zhang et al. 2018) as well as the *P. berghei* knock out screen (Bushell *et al.*, 2017; Schwach et al. 2015)) (see Table 7.6 and Table 7.7 in appendix and provided separately in excel file). This was combined with the whole proteome analysis showing a reduced abundance of six of those substrates. Those six NMT substrates were selected for further analysis of the essentiality of the N-terminal myristoylation. Three of them have been indicated (CDPK1, ARO) or proven (GAP45 (Perrin et al. 2018)) to be essential for the *P. falciparum* erythrocyte life cycle while S9C shows a slow growing phenotype (Zhang et al. 2018) and ISP3 (Poulin et al. 2013) was indicated to be dispensable. As previously mentioned, TRP has two paralogues, one being classified as dispensable (PF3D7_0601600) and the other as essential (PF3D7_0631000) (Zhang et al. 2018). For this screen PF3D7_0631000 was used. To study the essentiality of myristoylation, a CRIPSR-Cas9 screen was used to determine a ratio of integration of a G2G over a G2A mutation in each of the six substrates. For this Cas9 was used to generate a double strand break close to the N-terminal glycine of each of the six substrates using two different guides (Figure 3.13A) (see Table 2.4). Two repair plasmids were designed (see section 2.2.2.1) containing either an G2A point mutation preventing myristoylation or a G2G WT sequence using a different codon for glycine (Figure 3.13B). Additional sequence between the guide and the codon encoding the N-terminal glycine was recodonised, facilitating integration through homologous recombination. Both repair plasmids were mixed at a 50:50 ratio before linearization through restriction enzyme digestion at

locations flanking the two homology regions (Figure 3.13C). After transfection integration was facilitated through CRISPR-Cas9.

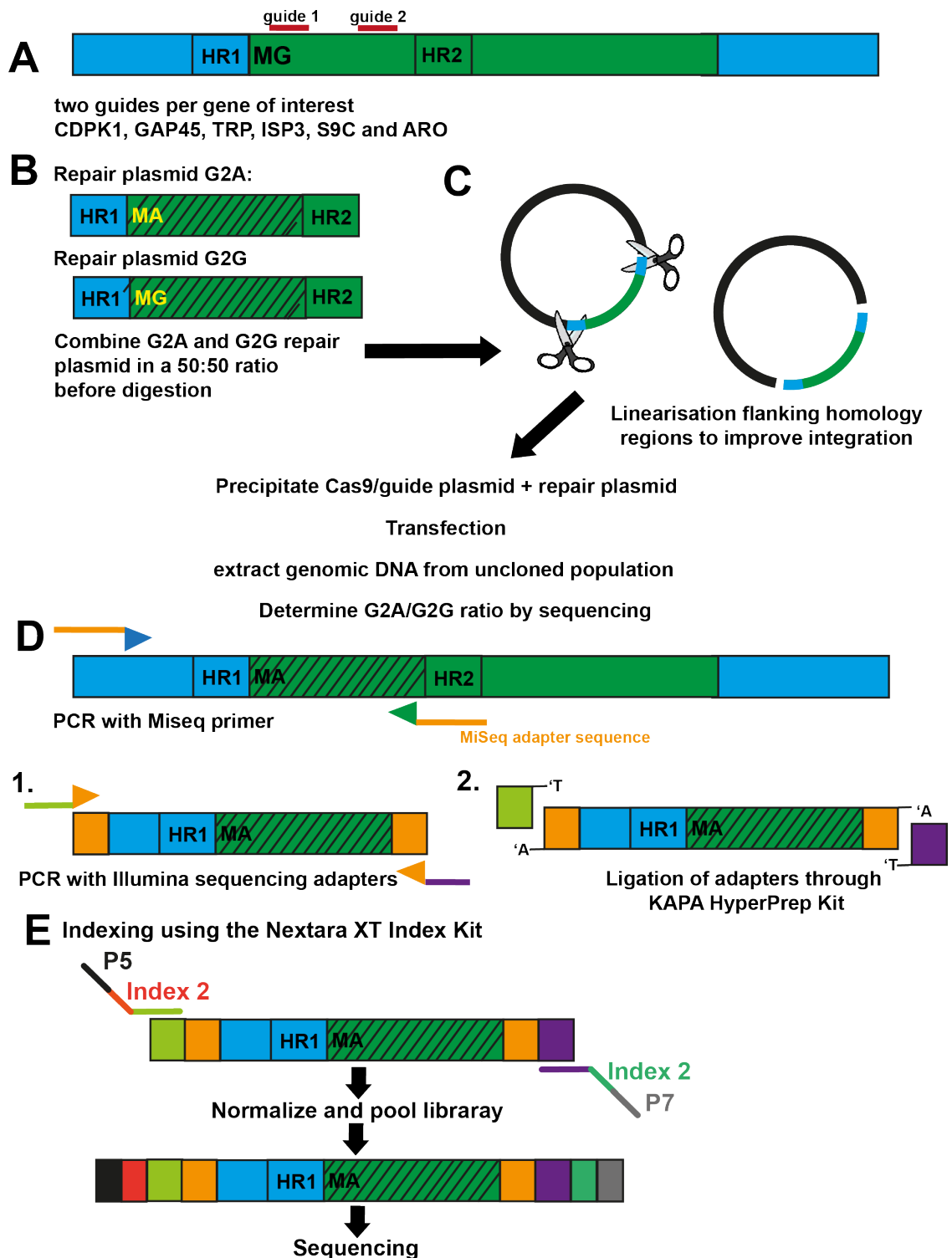


Figure 3.13 Illumina sequencing adapter addition through PCR or ligation.

A. For each gene of interest (CDPK1, ARO, S9C, GAP45, ISP3, and TRP) two guide sequences were selected (the same guide was used for both transfections for ARO as the first exon was too short to select two guides). B. Two repair plasmids with either a

G2A point mutation or a silent G2G mutation were generated. C. The plasmids were mixed at a 50:50 ratio before linearization flanking both homology arms. Integration was facilitated by CRISPR-Cas9 followed by genomic DNA extraction after successful transfection. D. Integration specific primer allowed a selective PCR of the integrated fragment, to which MiSeq adapter sequences were attached. The Illumina adapters were either attached through a second PCR using the MiSeq adaptors as primers (1) or through ligation using the KAPA HyperPrep Kit. This involves first repairing the overhangs of the PCR products to generate a blunt end, adding a dA-tail to the 3' which is then used as a binding site to for the Illumina-compatible adapters (2). D. To discriminate between samples, the adapter-ligated fragments are labelled with indices by indexing PCR with two indices at each end creating a unique barcode. This step also attaches the attachment site for the MiSeq instrument (P5 or P7). Following Illumina sequencing, the ratio of the number of sequences for either the G2A or G2G variant gives a measure of integration efficiency and an indication of the viability of either variant.

Genomic DNA was extracted and integration selective primers annealing to the recodonised sequence, were used to attach MiSeq adapter sequences for Illumina sequencing (Table 2.1) (see section 2.2.2.6). The Illumina adapters were added through a second PCR using the previously added MiSeq adapter sequences (Figure 3.13D) followed by indexing (Figure 3.13E) attaching unique barcodes to discriminate between sequences as well as attachment sites for the MiSeq instrument. The ratio of G2A/G2G of the sequence reads of each transfection gives an indication of the viability of the G2G variant over the G2A. For target sequence where no G2A variant was integrated there is a high probability of lethality if the substrate cannot be myristoylated. To search the sequence reads the first nine base pairs of the gene specific sequence which should mark the starting point of the sequence were used to filter out any sequence noise from the Illumina sequence library. A nine base pair sequence around the G2G WT sequence and G2A mutant sequence was used to determine the ratio of integration in the previously defined data set. For all genes, only the sequence from the reverse primer was used for the analysis due to very high AT-content in the 5' UTR, resulting in poor sequences coverage from the forward primer.

For five out of the six genes, there was >80% presence of the N-terminal glycine in the integrated construct with GAP45 and S9C showing nearly 100% (Figure 3.14A). For TRP the outcome was only 60% presence of N-terminal glycine while in 40% of the reads the N-terminal alanine was integrated. These results show that the N-terminal glycine is most likely essential for five out of the six substrates tested. For TRP it is difficult to draw any conclusions because of the presence of its paralogue.

Although, the second copy might be targeted by the same guide RNA because of the 99% sequence identity, both TRP genes would have been repaired through the second homology region downstream of the guide sequence. However, only the unique 5' UTR homology arm for PF3D7_0631000 would have facilitated the integration through double homologous recombination of either glycine or alanine codons from the recodonised repair construct. Therefore, the second TRP copy PF3D7_0601600 would be unchanged and might complement the lost function resulting from PF3D7_0631000 not being myristoylated.

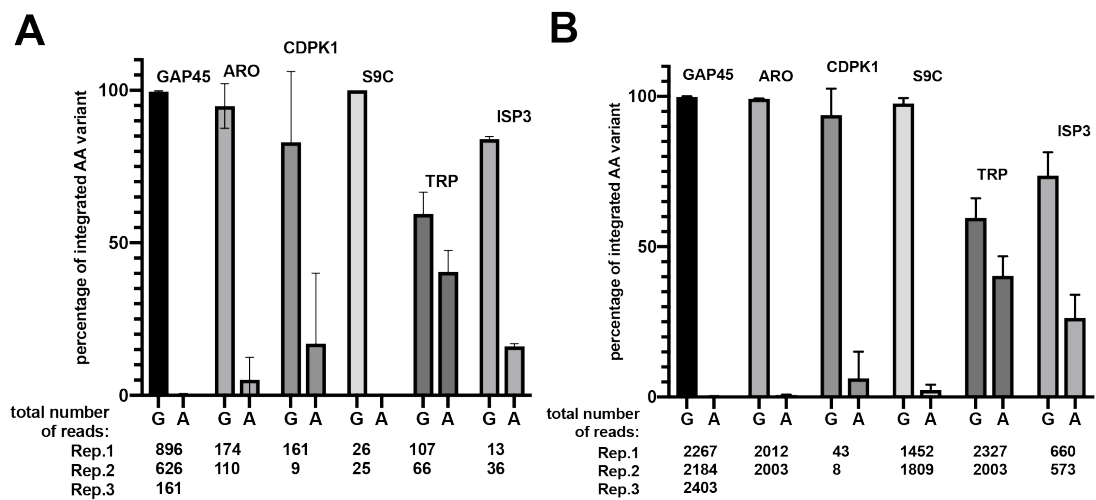


Figure 3.14 G2A/G2G competition viability screen.

G2A mutagenesis screen using CRISPR-Cas9 to insert G2A or G2G codons at the start of selected genes. Six genes were screened using a 50:50 ratio of two repair plasmids carrying a codon for G2G (G) WT (silent mutation) or for a G2A (A) point mutation. Illumina sequencing using integration specific PCR primers was used to determine the percentage of integration of each amino acid (AA) variant for each gene. Each gene was transfected at least twice (GAP45, n = 3) with two different guides (for ARO and GAP45 two transfections with the same guide were performed). The number of total sequence reads starting with the specific primer sequence used to amplify the integrated construct are indicated below the graph. A. Percentages of G2A vs G2G integration after the first sequencing using the PCR method to attached the Illumina sequencing adapters (see Figure 3.13D 1). B. Percentages of G2A vs G2G integration after a second sequencing using the ligation method to attach the Illumina sequencing adapter (see Figure 3.13D 2).

Overall the number of reads for all transfections containing the nine base pair starting point of the sequence was very low (between 9-896 reads per samples). The number of sequence reads was improved through attachment of the adapter sequences using ligation instead of a second PCR reaction (see section 2.2.2.6). This resulted in an up to 50-fold increase in number of sequences reads (Figure 3.14B). The

distribution of G2G and G2A incorporation, however did not change except for ISP3, with a 26% incorporation of the G2A mutation compared to 16% in the previous sequencing result (Figure 3.14). ISP3 was shown to be dispensable in *P. berghei* (Poulin et al. 2013), and only showed a slow growth rate in the *P. falciparum* knock-out screen (Zhang et al. 2018). It is therefore not surprising that parasites can tolerate some integration of a G2A variant leading to lack of myristoylation. Overall, the screen showed that for four out of six NMT substrates that myristoylation is likely essential (GAP45, ARO, CDPK1, and S9C), while for two substrates myristoylation might be dispensable (ISP3 and TRP).

3.7 Discussion

The phenotype of NMT inhibition is very variable and depends on the length of inhibition during the erythrocyte life cycle and the concentration of inhibitor used. Over 30 different NMT substrates have been identified in the erythrocyte life cycle stages and just over 100 proteins of the total Plasmodium proteome are predicted to be myristoylated (Wright et al. 2014). Many of the known NMT substrates are essential for the erythrocyte life cycle (see section 1.5) making it difficult to determine an underlying specific mechanism of NMT inhibition. The hypothesis for selective NMT inhibition for the entire erythrocyte stage was the failure to assemble the IMC during early schizogony, leading to a block in development, because key components of the IMC such as GAP45, ISP1 and ISP3 are not being myristoylated (Wright et al. 2014). Even though this might be part of the developmental blockage, it most likely is a consequence of earlier events happening due to NMT inhibition. GAP45 expression only starts just before 33 h PI (Ridzuan et al. 2012) and parasite death due to NMT inhibition from ring stage, occurs around 35-37 hours with 4-5 nuclei (Wright et al. 2014). In those first 33 h of the life cycle, presumably other NMT substrates not being myristoylated might initiate the pleiotropic consequences. When the drug inhibition window is reduced to the last eleven hours, parasites develop to morphologically fully mature schizonts but the events leading on to egress are inhibited leading to a significant drop in parasitaemia in the subsequent cycle. Most of the identified substrates are highly expressed in those last eleven hours of the erythrocyte life cycle (Wright et al. 2014). Analysis of the phenotype indicated no

observable changes on Giemsa images. However, immunofluorescence images showed that localisation of some NMT substrates such as ARO is impaired, not localising to the rhoptries. IMC formation and localisation of micronemal markers, on the other hand, seemed normal. Since ARO may be involved in the correct localisation of rhoptries at the apical end of developing merozoites by homology with *T. gondii* (C. Mueller et al. 2016), NMT inhibition might lead to mislocalization of rhoptries. This mislocalisation of rhoptries also was observed after treatment with the palmitoylation inhibitor 2-bromopalmitate (2-BP) during the last 24 hours of the erythrocyte life cycle (Jones et al. 2012), although interpretation of these data requires caution because this compound has many off target making it difficult to discern the cellular outcome of loss of palmitoylation (Lanyon-Hogg et al. 2017; Davda et al. 2013). ARO has two cysteines (C5 and C6) which are likely palmitoylated and are involved in membrane anchoring and therefore rhoptry positioning, together with the N-terminal myristoylation. With 2-BP treatment rhoptries formed but are in aggregates or not enclosed within a specific developing merozoite structure which was analysed by electron microscopy (Jones et al. 2012). The work carried out by Cabrera et al., on episomal expressed ARO_{A2} and ARO_{A5A6} mutants showed a change in localisation from the apical rhoptries to the cytosolic (Cabrera et al. 2012). These findings support the observation in this thesis that loss of myristoylation of ARO changes the localisation of the protein which may indicate that rhoptries might not be localized at the apical end of merozoites during schizogony after NMTi treatment.

To determine the change in *N*-myristoylation as well as whole protein abundance after NMT inhibition, two proteomic experiments were performed indicating that some NMT substrates respond differently to NMT inhibition. Through a competitive proteomic experiment enriching for proteins modified by YnMyr, sixteen proteins showed a significant reduction in *N*-myristoylation after NMT inhibition. Due to the co-translational nature of *N*-myristoylation, only those proteins being synthesized during the YnMyr labelling and NMT inhibition window will be detected and pulled down. The hypothesis is that proteins showing a strong change in probe labelling efficiency respond strongly to NMT inhibition and might contribute more to the observed phenotype. Six of those substrates also show a significant difference in overall protein abundance after NMT inhibitor treatment in saponin lysed parasites.

This led to the hypothesis that NMT inhibition might affect their overall stability. This is similar to what has been observed with parasites treated with palmitoylation inhibitor 2-BP from 42-48 h PI, with a significant effect on stability of proteins such as palmitoylated GAP45 and MTIP (Jones et al. 2012). NMT substrates are not the only proteins decreasing in abundance as a response to NMT inhibition. There was also a significant downregulation of proteins involved in DNA chromatin organization and assembly such as histone proteins, and proteins involved in DNA replication, translation and transcription. The second largest group involved a downregulation of exported and secreted proteins especially some to the erythrocyte membrane. The difficulty interpreting this data set lies in the observed protein abundance being a result of changes in transcription levels as well as considering protein stability involving protein degradation rates. An additional challenge comes from only being able to detect about 46% of the total life cycle proteome (2484 proteins out of 5400) through the MS data, resulting from loss of proteins present in earlier life cycle stages and during lysis conditions and a bias of some peptides behaving better through mass spectrometry analysis than others. Expression of most exported parasite proteins to the RBC peaks in early erythrocyte asexual stages, but some proteins are also expressed and stored in the apical organelles before invasion (Marti et al. 2005). There might be a downregulation of some exported proteins due to loss in the saponin lysis processing step, which is why they are not downregulated in the whole proteome before saponin lysis. Another difficulty with NMT inhibition is the delay in development. Although schizonts are able to generate an equal number of nuclei compared to DMSO treated parasites, after the eleven-hour drug inhibition, NMT inhibition might still slow down many essential processes during schizogony with parasites being less mature and leading to changes in protein abundance.

Total protein synthesis accelerates at around 18 – 24 h PI (Holder & Freeman 1982) and late schizonts are at the peak of protein synthesis and DNA synthesis, starting to segment into individual merozoites which includes a high expression of core histone protein coinciding with DNA synthesis (Miao et al. 2006). During schizogony, NMT inhibition has the most profound impact with the shortest inhibition time. The “pseudoschizont” phenotype observed after 48 hours of inhibition, presents a lag phase until parasite death is observed at around 36 – 38 h PI while an eleven-hour window kills parasites in a much shorter time. Since *N*-myristoylation is a co-

translational process, it raises the possibility that NMTi and protein synthesis inhibition phenotypes may be connected through common essential downstream factors. In the whole proteome analysis, proteins involved in DNA synthesis, transcription, translation and chromatin organisation were less abundant as a response to NMT inhibition strengthening the hypothesis of a connection between DNA and protein synthesis and NMT inhibition. Furthermore, there might be a correlation of NMTi acting most profoundly when protein synthesis accelerates leading to a lag phase during ring and early trophozoites where protein synthesis is low, but acting faster during schizogony. Wright *et al.* showed that total protein synthesis is not directly affected by NMTi by showing no effect on tagged methionine surrogate azidohomoalanine (AHA) using protein synthesis inhibitor cycloheximide (CHX) as a control (Wright *et al.* 2014). This is expected for a selective inhibitor of NMT, implying that the initial MoA is distinct (Wright *et al.* 2014). Many protein synthesis inhibitors stop parasite development in the transition from ring to trophozoite/schizont stage at the point where protein synthesis starts to increase. For example, Baragaña *et al.* showed that the translation elongation factor 2 (eEF2) inhibitor DDD107498 prevents trophozoite and schizont development, generating morphologically abnormal schizonts when treatment is commenced at ring stage (Baragaña *et al.* 2015). Images from Giemsa-stained thin blood smears suggest that parasites arrest before DNA synthesis starts at around 24 h PI, and schizont formation was prevented, causing a 50% reduction in parasitaemia. It is possible that NMTi may in some way resemble inhibitors of a subset of protein synthesis, which would explain a similar phenotype. The parasite response to nutrient restriction, which in some ways phenocopies the effect of protein synthesis inhibition, is a universal evolutionary adaptation and may drive the process of differentiation into gametocytes (Ono *et al.* 1993). The efficacy of NMT inhibition in other stages of the life cycle has not been reported to date, but it will be interesting to see whether NMTi mimics the same broad spectrum of activity as DDD107498 throughout the life cycle.

In addition to the possible protein mis-localization (as observed for ARO), the downstream consequences of NMT inhibition may also include protein misfolding, leading to protein degradation resulting in the observed decrease in protein abundance of some NMT substrates. In several types of cancer cell, NMT inhibition leads to cell death through apoptosis during G1 phase, potentially as a result of ER

stress (Thinon et al. 2016). It has been proposed that inhibition of *N*-myristoylation leads to an accumulation of mis-localized proteins that are degraded by the proteasome. Furthermore, the *Plasmodium* 26S proteasome regulatory subunit 4 (PF3D7_1008400) is itself an NMT substrate (Wright et al. 2014) and malfunction of this subunit due to lack of *N*-myristoylation might reinforce the ER stress response.

Inhibition of NMT may also lead to an imbalance in favour of other N-terminal protein modifications (NPMs) such as *N*- α -acetylation (NAT) carried out by N-terminal acetyltransferase (NATs) (Starheim et al. 2012), or N-terminal ubiquitination (Ciechanover & Ben-Saadon 2004). Usually these NPMs occur co-translationally through ribosome-associated protein biogenesis factors (RPBs) that interact with the ribosome and show a degree of competition in their binding (Giglione et al. 2015). For example, there is already some indication of competition between *N*- α -acetylation and *N*-myristoylation (Utsumi et al. 2001).

Because of the large number of myristoylated proteins, it is very difficult to draw conclusions as to which of the substrates has a larger impact on the phenotype. Using an NMT inhibitor to study its function and the resulting phenotype results in pleiotropic consequences with all substrates effected at the same time. In order to study *N*-myristoylation in isolation on particular substrates, the proteomic data sets were used to select six NMT substrates for a G2A substitution screen to determine the essentiality of the N-terminal glycine. The hypothesis is not that any of those G2A substrates would phenocopy the NMT inhibition phenotype but rather show that *N*-myristoylation in isolation on a particular substrate is essential. The screen showed that four out of the six proteins show >80% incorporation of the N-terminal glycine in the integrated construct with GAP45, ARO and S9C showing nearly 100%. This strongly suggest the essentiality of myristylation for at least four of the tested substrates.

Chapter 4. Conditional Glycine to Alanine substitution of NMT substrates

The proteomic experiments carried out in section 3.3 helped to determine NMT substrates that have significantly reduced incorporation of YnMyr with the NMT inhibitor IMP-1002 present. The data on the changes in protein abundance levels after NMT inhibitor treatment (see section 3.4), helped to select NMT substrates that were significantly reduced in abundance in saponin treated samples. This led to the hypothesis that NMT inhibition might affect the protein stability of some NMT substrates. Furthermore, IFA images showed that substrates are not targeted to their normal membrane location leading to their mislocalisation. This was the case for the rhoptry protein ARO for example. All NMT substrates were analysed for their essentiality in *P. falciparum* using the data from a recent insertional mutagenesis screen (Zhang et al. 2018) as well as the knowledge acquired from a global screen in *P. berghei* (Bushell *et al.*, 2017; Schwach et al. 2015). Findings were combined in Table 7.6 and Table 7.7 (also available in separate excel file) and six NMT substrates were selected for a glycine to alanine screen (G2A screen). The G2A screen was a competition screen between the integration of an N-terminal glycine and an N-terminal alanine construct to repair a double strand break induced by CRIPSR-Cas9 in the six selected substrates. The incorporation of the N-terminal glycine [G2G] over the N-terminal alanine [G2A] indicates the likely essentiality of the N-terminal glycine and therefore myristoylation of those six substrates (see section 3.6). The screen showed that myristoylation is likely essential for four out of the six substrates (Figure 3.14). This included GAP45 and ARO which have been selected to be further analysed using a conditional G2A approach to study the phenotype of the lack of myristoylation on NMT substrates in isolation.

The *P. falciparum* asexual blood stage genome is haploid preventing the establishment of parasite lines with mutations or deletions in essential genes. The likely essentiality of the six genes in the G2A screen was therefore solely determined by the lack of integration and establishment of the [G2A] construct and the successful integration of the [G2G] construct. It does not however, allow any phenotypic characterization of the parasites lacking the N-terminal glycine. Therefore, the DiCre

system (see section 2.2.1.6) in which the enzyme Cre recombinase is expressed as two enzymatically inactive domains, was used to generate a conditional G2A substitution mutant of two NMT substrates (GAP45 and ARO). Upon addition of rapamycin, the two inactive Cre domains heterodimerise and form a fully functional and active enzyme, which catalyse excision of the DNA sequence between two loxP sites excising the [G2] copy of the NMT substrate bringing the [G2A] copy into place for expression from the promoter (Figure 4.1). The integration of the repair plasmid with the region of interest is achieved by utilising the absence of non-homologous end joining (NHEJ) in *P. falciparum* to allow double strand (DS) breaks induced by Cas9 to be repaired only by the “error-free” homologous recombination (HR) pathway (Lee et al. 2014) (Figure 4.2). During HR, the two homology regions on the repair plasmid will serve as templates for highly accurate repair through double HR. The strand invasion during the double crossover event results in a D-loop which is resolved through synthesis dependent strand annealing, which displaces the sequence in between the homology arms in the genome during the repair mechanism (Lee et al. 2014) (Figure 4.2).

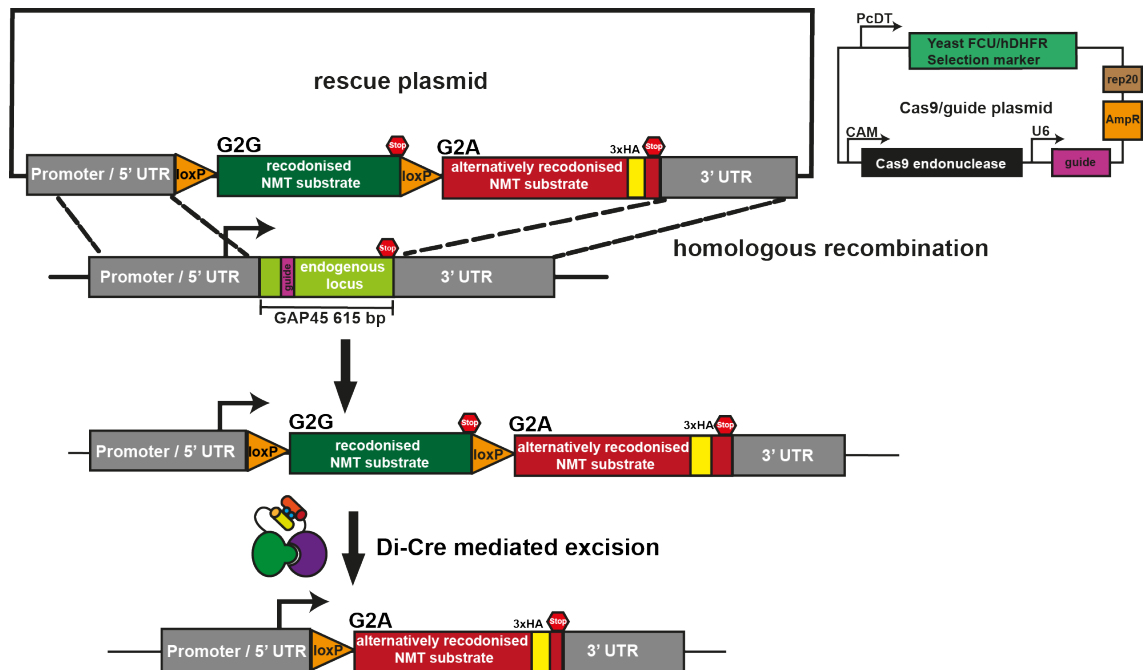


Figure 4.1 Conditional G2A mutation strategy.

Two loxP sites flank the recodonsed wild type G2 copy of the NMT substrate gene followed by a second alternatively recodonsed gene copy starting with the G2A mutation and tagged with an HA tag. Upon addition of rapamycin, the two Cre recombinase fragments dimerize and form an active DiCre enzyme which will excise the wild type G2 NMT substrate copy and bring the second G2A copy upstream of the promoter to be expressed.

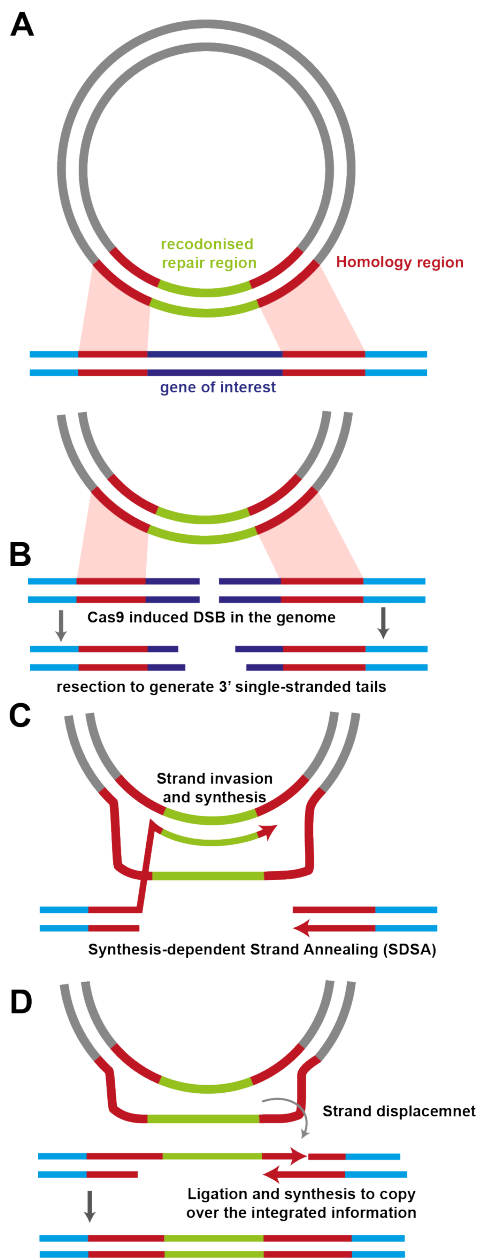


Figure 4.2 Homologous recombination (HR) following CRISPR-Cas9 mediated double strand break (DSB).

A. During HR the two homology arms on either side of the recodonised repair region serve as homology templates to repair the DSB induced by Cas9. B. The DSB is followed by resection of the dsDNA to generate 3' single-stranded tails. C. Strand invasion into the repair plasmid allows the synthesis from the repair construct by synthesis-dependent strand annealing (SDSA). D. Strand displacement, ligation and synthesis of the reverse strand.

Previous work to study the essentiality of myristoylation were focused on CDPK1 and GAP45. As outlined in section 1.5.1, the IMC-associated NMT substrate GAP45 plays an essential role in the invasion mechanism of the parasite (Perrin et al. 2018). Studies have shown that a chimeric GFP fusion construct of the first 48 amino acids of CDPK1 containing N-terminal myristoylation, palmitoylation and a basic cluster, localized to the IMC (Möskes et al. 2004). A myristoylation-negative mutant (G2V), in which Gly at position 2 was replaced by a Val, reduced membrane binding to 28%. A deletion mutant (D10-20) which lacked the polybasic cluster reduced membrane binding to 32% and a double mutant (DM) lacking both features reduced the membrane binding to 7%.

Building on the work in Möskes et al., the Holder lab looked at a chimeric GFP fusion construct of the N-terminus of GAP45 (Ridzuan et al. 2012) Rab5b (Ezougou et al. 2014) and AK2 (Thavayogarajah et al. 2015) (see section 1.5.2) through fluorescence microscopy and a fractionation assays. Just as for CDPK1, GAP45, Rab5b and AK2 GFP chimera proteins require the myristoylation site for correct localisation, because a G2A mutant does not localise at the IMC and is found in the cytosol as a soluble protein, as evidenced by being present in the hypotonic lysis fraction of schizonts rather than in the carbonate pellet as for the WT GFP chimera. The N-terminal

palmitoylation site of Rab5b at position C3 was also shown to be essential, showing the same phenotype as the G2A chimeric GFP fusion protein, by being mis-localised to the cytoplasm as detected using an indirect IFA and being present in the hypotonic lysis fraction (Ezougou et al. 2014). For GAP45, the deletion of the palmitoylation site at position C5 has a negative effect on the GFP chimeric fusion protein changing its location to partially cytosolic and a presence in multiple fractions including the hypertonic soluble, high salt soluble, carbonate soluble and the carbonate insoluble pellet (Ridzuan et al. 2012). Also for AK2, the lack of palmitoylation at C4, led to only a small fraction being associated with the membrane and the protein not being secreted beyond the PPM (Thavayogarajah et al. 2015). For all of these proteins, the GFP fusion construct is only targeting the N-terminus and the approach does not consider any palmitoylation sites at the C-terminus. This is particularly important for GAP45 which has five predicted palmitoylation sites at the C-terminus. It is, therefore, important to study an endogenous full length G2A variant protein, which will also allow observation of the phenotype resulting from loss of myristoylation.

This chapter will describe the difficulties involved in creating and analysing the conditional G2A substitution in the *GAP45* and *ARO* gene, which resulted mainly from the placement of the loxP site in front of the ATG start codon being fatal for parasites, leading to a selection of parasites in which a short stretch of homologous DNA of 8-12 base pairs was used for recombination, losing the loxP site. It will further describe an alternative complementation strategy in which a conditional knock out of the *GAP45* gene allows the conditional replacement with a second copy in which sequence for the N-terminal glycine is replaced by that for alanine.

4.1 Partial integration of *GAP45*[G2A] and *ARO*[G2A] due to lethality of loxP directly upstream of the start codon.

The failure of integration of the G2A construct into both *GAP45* and *ARO* genes in the G2A Screen (see section 3.6), was used as an indication of the essential nature of the N-terminal glycine and therefore of myristoylation. Both substrates were selected for a conditional G2A modification, because of their essential nature for the parasite, their change in location (*ARO*) after NMTi treatment, the readily available

antibodies for both, and their strong response in myristoylation pattern and protein abundance after NMTi treatment. It was decided therefore to exploit the DiCre system, which has recently been adapted for use in *P. falciparum* (Collins, Das, et al. 2013; Knuepfer et al. 2017). This approach would have the advantage of being able to confirm the unambiguous essentiality of myristoylation for GAP45 and ARO and to investigate the phenotypic consequences of the lack of myristoylation of those two selected substrates.

The genetic modifications were made in the p230p DiCre parasite clone (Knuepfer et al. 2017). The clone constitutively expresses the Cre recombinase enzyme components which form an active recombinase upon the addition of rapamycin and will excise the region of DNA between two loxP sites ('floxing'). After excision of the NMTs[G2] copy of *GAP45* and *ARO*, the NMTs[G2A] copy will be positioned behind the endogenous promoter to be expressed (Figure 4.1). The two loxP sites need to flank the entire *GAP45*[G2] copy including the ATG encoding the initiator methionine and the following glycine codon. This resulted in the strategy of placing the 34 bp loxP site directly upstream of the initiator ATG. As discussed in section 1.9.2, several attempts to place the loxP site at various distances from the ATG have been made in other organisms (Drewry & Sibley 2015; Weissman & Pan 2015). However, there has been no successful integration of the loxP directly upstream of the ATG or within the promoter region reported in *P. falciparum*.

4.1.1 The lethality of the loxP site directly upstream of the start codon in the conditional *GAP45*[G2A] line

As described in section 2.2.2.2, the Δ *GAP45* construct *gap45:ha3:loxP* provided by Abigail Perrin (Perrin et al. 2018), was used to generate a *gap45:loxP::gap45[G2G]:ha3* and *gap45:loxP::gap45[G2A]:ha3* repair constructs. The guide was provided by Abigail Perrin on a pDC2-cam-Cas9-U6-hDHFRyFCU-plasmid (Knuepfer et al. 2017; Lim et al. 2016; Macpherson & Scherf 2015) and had previously been used successfully to generate the Δ *GAP45* line (Perrin et al. 2018). The two repairs plasmids were paired with the guide-Cas9 plasmid and transfected on two independent occasions. Selection of the

guide-Cas9 plasmid was achieved through expression of the human *dhfr* gene, which was selected using 2.5 nM WR added at ~24 h post infection and renewed daily for two asexual cycles (four days). Parasites were first observed after 15-19 days after transfection and PCRs using primer that amplify across the 3' and 5' integration site were used to determine integration efficiency of all four transfections (Figure 4.3).

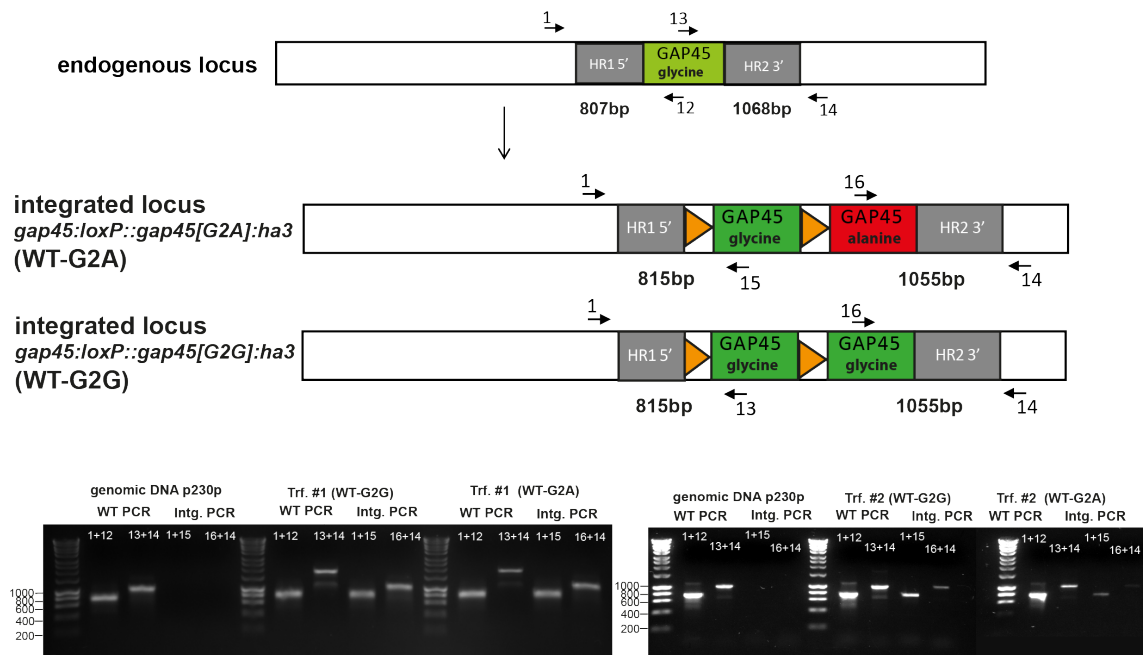


Figure 4.3 Integration PCR of parasites transfected with *gap45:loxP::gap45[G2A]:ha3* and *gap45:loxP::gap45[G2G]:ha3*.

Integration was detected with specific primers (Intg. PCR: 1 + 15 and 16 + 14) at the 3' and 5' ends of the integrated construct, while another set of specific primer amplify only from the endogenous unmodified locus (WT PCR: 1 + 12 and 13 + 14). Genomic DNA from the p320p DiCre expressing line used for transfection was used as control for the WT-specific primers. The ratio of band intensity from the WT and Intg. PCR between the two transfections indicates the ratio of integrated vs WT parasites in the transfected population. Overall there was a higher integration frequency in the first transfection.

Then all four lines were cloned by limiting dilution. One clone from each transfection was selected for further investigation and integration was again confirmed through PCR (Figure 4.4) resulting in the validation of two clones carrying the *gap45:loxP::gap45[G2G]:ha3* and two carrying the *gap45:loxP::gap45[G2A]:ha3* construct.

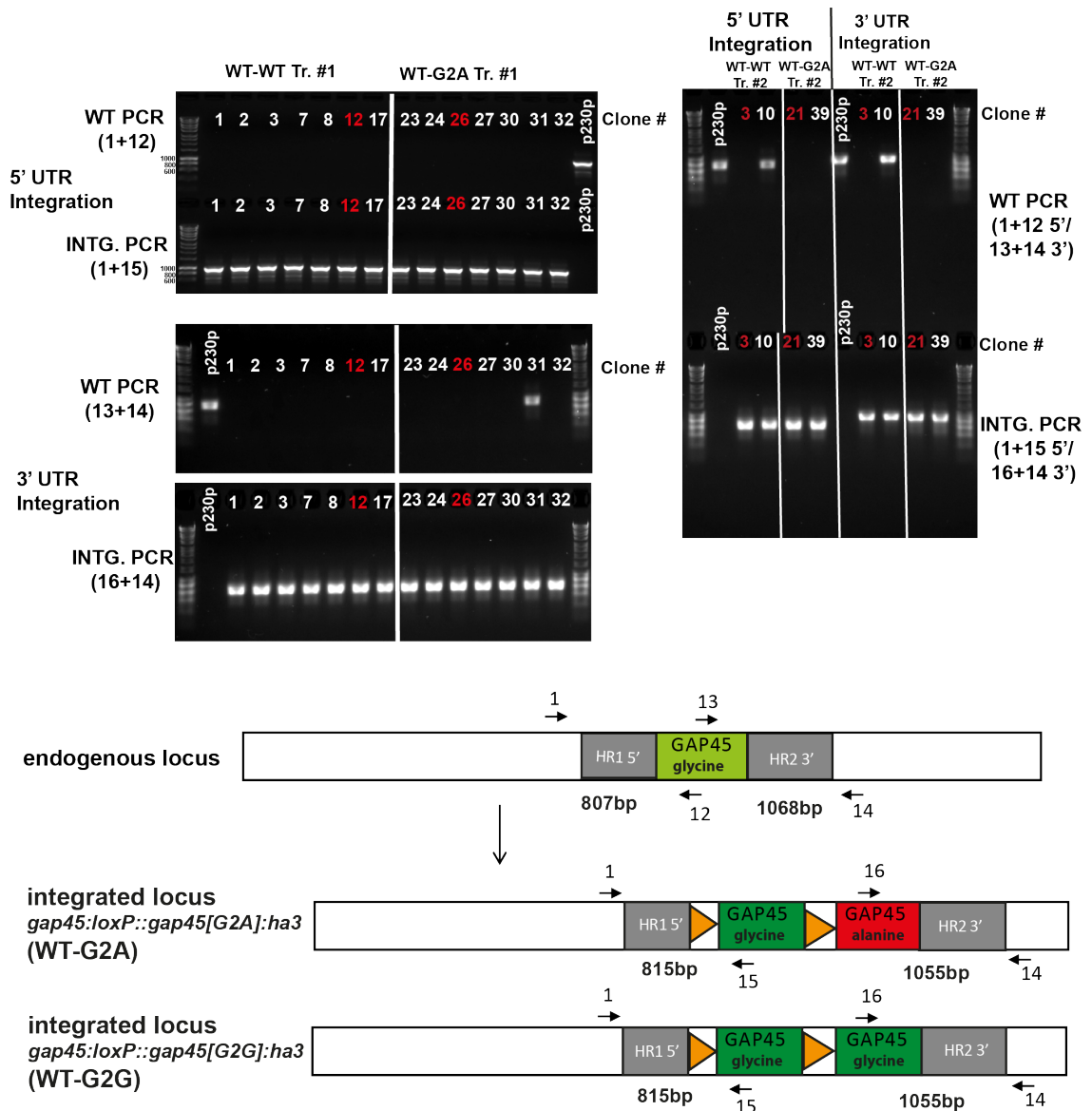


Figure 4.4 Integration PCR of transfected clones with the *gap45:loxP::gap45[G2A]:ha3* and *gap45:loxP::gap45[G2G]:ha3* constructs.

Integration was detected with specific primers (1 + 15 and 16 + 14) at the 3' and 5' end of the integrated constructs (bottom gel) while the second set of PCR reactions was used in an attempt to amplify from the endogenous wild type locus (1 + 12 and 13 + 14) (top gel). Only in one clone was there still detection of endogenous wild type DNA. Clones coloured in red were selected for further analysis.

To determine if excision of the *gap45:loxP* would occur, selected clones were treated with rapamycin (see section 2.2.1.6), genomic DNA extracted (see section 2.2.1.3) and two specific primer combinations were used for PCR as outlined in Figure 4.5. The difference in the primer combinations was in their location relative to the integrated region. While primers 1 and 10 are present within the homology region, primers 11 and 14 are outside of the homology region (Figure 4.5A).

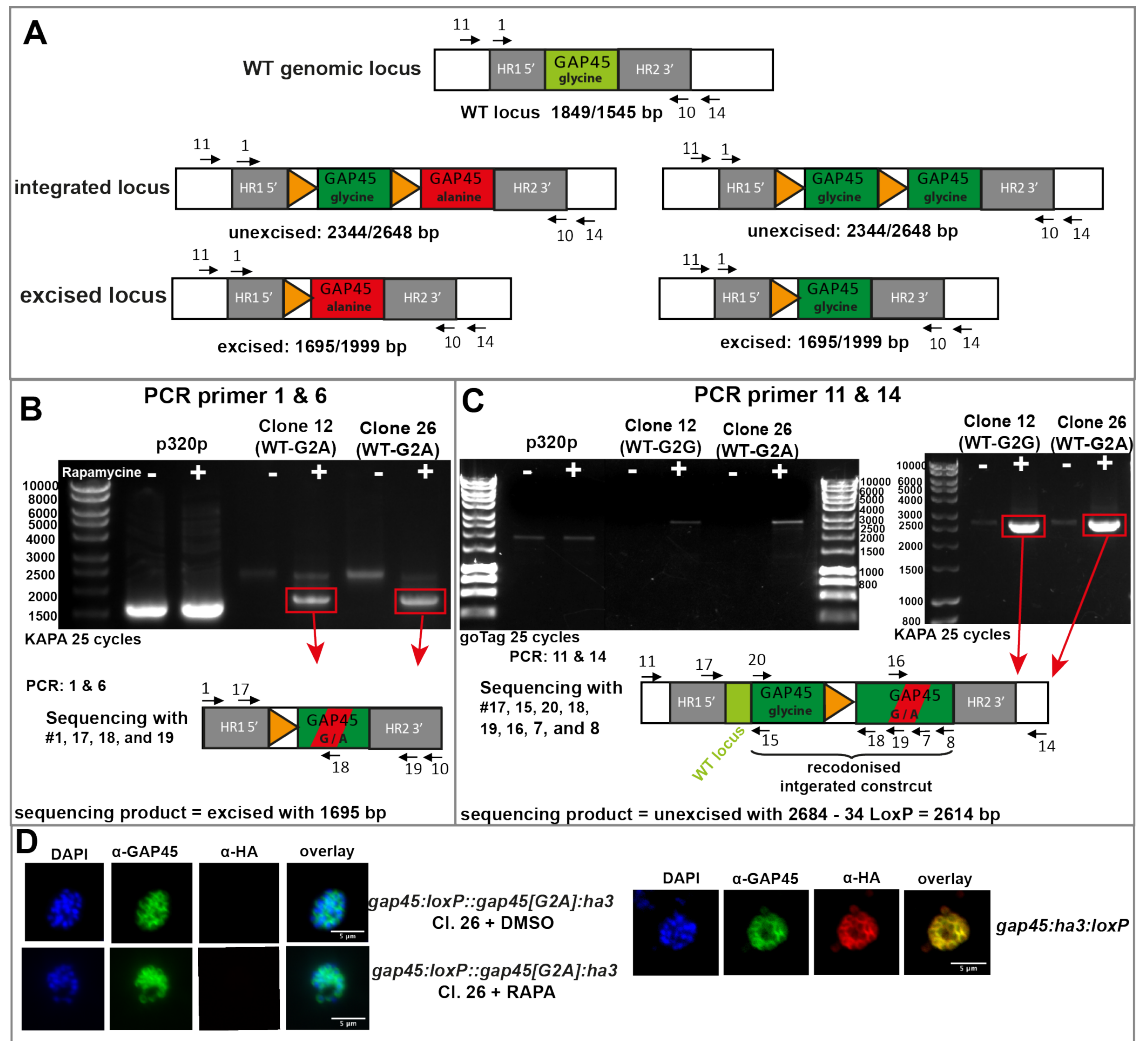
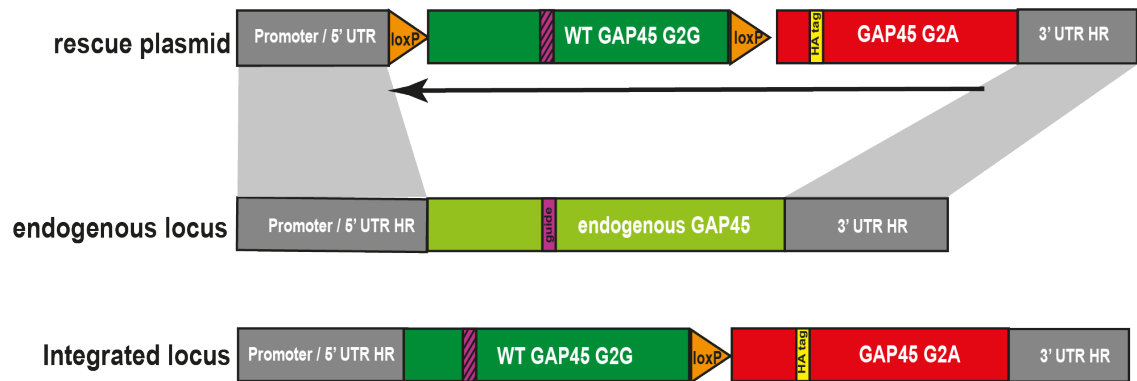


Figure 4.5 Integration occurred through homologous recombination within a 12-13 bp stretch of DNA downstream of the loxP site.

This homologous recombination event prevented integration of the loxP site directly upstream of the ATG start codon. A. *gap45:loxP::gap45[G2A]:ha3* and *gap45:loxP::gap45[G2G]:ha3* constructs indicating two sets of primer within and outside of the homology regions. B. Excision PCR with the primer pair within the homology arm and indicating partial excision after rapamycin treatment. Sequencing result of the lower band (red box) is visualised below for both for the *gap45:loxP::gap45[G2G]:ha3* (green Clone 12) and *gap45:loxP::gap45[G2A]:ha3* (red Clone 26) constructs. Both clones show the same excised sequence with either a G2G or G2A codon (green/red mixed). C. Excision PCR using primers outside of the homology regions using two different polymerases for amplification. PCR using outside primers indicates no excision. Only KAPA polymerase is able to amplify from the DMSO control. Sequencing of the PCR product after rapamycin treatment (red box) revealed that the integrated construct contained the sequence of the endogenous wild type locus for the first 60 bp (WT-G2G)/125 bp (WT-G2A) (light green) followed by the remaining construct. The loxP site directly upstream of the start codon was missing. D. Immunofluorescence images showing no HA staining, indicating no excision. *gap45:ha3:loxP* parasites, expressing HA tagged GAP45 were used as a positive HA control.

A PCR with primers 1 and 10 suggested a partial excision in both clones (Figure 4.5B). The lower band was sequenced and it revealed an excised product containing the loxP site followed by *gap45[G2A]:ha3* / *gap45[G2G]:ha3*. However, when the PCR was performed using the two outside primers 11 and 14, it revealed a full-length band that had not been excised by DiCre in both clones using GoTaq following rapamycin treatment (Figure 4.5C). Surprisingly no PCR product was amplified from DMSO-treated control parasites. A change in polymerase to KAPA showed that from both rapamycin and DMSO control, a full-length unexcised PCR product of 2650 bp was amplified which is the expected size of 2684 bp minus one loxP site of 34 bp. The sequencing revealed that a region of 60 base pairs for clone 26 and 158 bp for clone 3, 12, and 21 (clone 3 and 21 from the second transfection are not shown in Figure 4.5, is not recodonised but identical to the endogenous WT sequence. A stretch of 12 bp (clone 26) and 11 bp (clone 3, 12, and 21) was identical in the endogenous WT copy and the recodonised construct. Usually the sequence identity between the endogenous copy and the rescue construct is kept to a minimum but on this occasion a longer stretch was necessary to provide enough sequence dissimilarity between the two *GAP45* copies to prevent an additional homologous recombination event between two potential identical copies. The results showed that all four clones used a stretch of 11 to 12 bp for homologous recombination to repair the double strand break induced by Cas9 using the template available, and resulting in the loss of the first loxP site directly upstream of the ATG start codon (Figure 4.6). Because only one loxP was successfully integrated, excision cannot occur. This result was further confirmed through an indirect IFA (see section 2.2.3.1) to detect the HA tag in the second copy of *GAP45* downstream of the second loxP site (Figure 4.5D). Neither in the DMSO-treated control nor the rapamycin treated clone 26 parasite line (*gap45:loxP::gap45[G2A]:ha3*) was the HA tag detectable, indicating no excision. The *gap45:ha3:loxP* line provided by Abigail Perrin (Perrin et al. 2018) was used as a control and this showed a positive signal of the HA tag that was co-localised with the anti-GAP45 antibody. In summary, in all four clones from two independent transfections the first loxP site was not integrated resulting in a construct that cannot be excised. This occurred through a homologous recombination event using 11 to 12 bp (Figure 4.6).

A



B

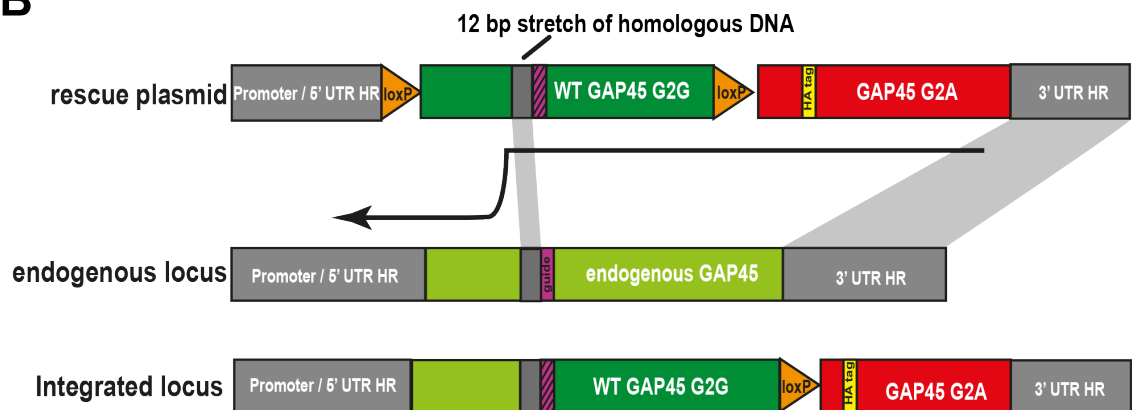


Figure 4.6 Selection pressure of the lethal loxP directly upstream of the start codon leads to homologous recombination through a 12 bp DNA sequence.

A. The stretches of 200bp homology regions (grey) on each side of the GAP45 endogenous target sequence (light green) were designed to facilitate integration of the rescue construct (green/red). B. A 12 bp homology region close to the guide cutting sequence (purple) facilitated integration of part of the rescue construct but prevented integration of the loxP site directly upstream of the ATG start codon. This resulted in the construct sequence being integrated only after the 12 bp homology region

To answer the question why rapamycin treatment appeared to result in partial excision as judged using primers amplifying within the homology region (Figure 4.7), a possible retention of the rescue plasmid was investigated by PCR. This was done using primers 21 and 22 (combination A) that amplify from the pL6MCSminusDHFRU6 backbone used for cloning the *gap45::loxP::gap45[G2A]:ha3* or *gap45::loxP::gap45[G2G]:ha3* constructs. Both clone 12 and 26 still contained plasmid DNA as indicated by the bands from PCR reactions A, B and C (Figure 4.7). Clones 3 and 21 on the other hand showed no sign of residual plasmid. The only difference in the two transfections as stated in section 2.2.2.2, was the usage of

different restriction enzymes to linearize the rescue plasmid: PstI (cut sequence: CTGCAG, 3' overhang, transfection #1, clone 26, 12) or Scal (cut sequence: AGTACT, blunt cutter, transfection #2 clone 03, 21). Both cut sites are over 1000 bp away from the homology arms and should not have resulted in any issues of plasmid retention. Only circular plasmids can be maintained as self-replicating episomal concatemers in *P. falciparum*, whereas linear DNA is lost after about four days (Deitsch et al. 2001); therefore, the only possible explanation is that the plasmid re-circularised with PstI, but not with Scal. Both restriction enzyme digests were analysed for their successful digestion on an agarose gel prior to transfection. However small amount of undigested material for the PstI cut construct might have been overlooked.

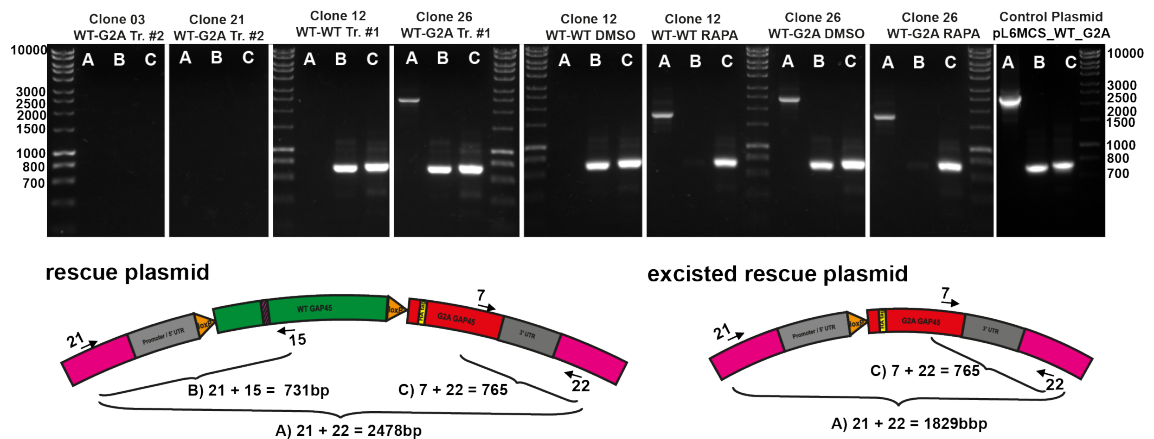


Figure 4.7 Some clones still contain the rescue plasmid from which successful DiCre mediated excision can occur.

To show the presence of the rescue plasmid, three primer combinations were used (A, B and C). Only primer combinations A and C can anneal to the excised rescue plasmid sequence due to the missing sequence of *gap45:loxP* where primer 15 anneals. For both clones from transfection #1 there was a positive band after PCR reactions for at least two of the three primer combinations, indicating the presence of the plasmid. After rapamycin treatment successful excision from the plasmid was indicated by a shorter band from primer combination A, while B no longer generated a PCR product. The plasmid used for transfection was used as a positive control. Clones from transfection #2 did not show positive PCR reactions indicating that the plasmid was absent.

4.1.2 The lethality of the loxP site directly upstream of the start codon in the conditional *ARO*[G2A] line.

As described in section 2.2.2.2, the *aro:loxP::aro*[G2G], *aro:loxP::aro*[G2G]:*ha3*, *aro:loxP::aro*[G2A] and *aro:loxP::aro*[G2A]:*ha3* repair construct sequences were commercially synthesised from Gene Art and combined with PCR-amplified 5' and 3' homology regions as well as sequences encoding a stop codon and HA3 tag (Figure 4.8A). Two different sgRNAs were used, targeting Cas9 to a sequence in the first and the last exon (exon 11) (see section 2.2.2.2). Following transfection, parasites were detectable after 22 to 25 days. For one transfection (*aro:loxP::aro*[G2A]:*ha3* with Guide 2) no parasites survived following transfection, and for two transfections electroporation failed (*aro:loxP::aro*[G2G] and *aro:loxP::aro*[G2A] with Guide 1). From the remaining 5 successful transfections, genomic DNA was extracted to use for a PCR-based integration screen (Figure 4.8A, XB). Primer pairs amplifying regions incorporating the 3' and 5' integration sites for the WT genomic locus, as well as primers for the four different integrated constructs, were used to determine the ratio of WT versus integrated sequence in the transfected population. The PCR screen showed that only two out of the 5 transfections revealed evidence of successful integration of the rescue plasmid: *aro:loxP::aro*[G2A] (WT-G2A) and *aro:loxP::aro*[G2G]:*ha3* (WT-WT_HA3), both with Guide 2, and indicated by at least one out of the three integration PCRs (C, D and E) showing a visible band. PCR reaction C was expected to give a fragment of 1164bp which was not the case in any of the transfections and reaction D only produced a product for *aro:loxP::aro*[G2G]:*ha3* (WT-WT_HA) but with an additional ~700 bp in size. The genomic DNA from *aro:loxP::aro*[G2G]:*ha3* was used for sequencing to determine whether the first LoxP site directly upstream of the ATG had been integrated successfully. As indicated in Figure 4.8C, a stretch of 8 bp on the rescue plasmid had served to provide homologous recombination with the genomic locus. Similar to the *gap45:loxP::gap45*[G2A]:*ha3* and *gap45:loxP::gap45*[G2G]:*ha3* transfections, a small stretch of DNA had been used under the selection pressure of the lethal loxP site directly upstream of the ATG, to repair the double strand break induced by guide 2. This resulted in only a partial integration of the rescue plasmid with the first

six introns remaining in the repaired locus. Furthermore, due to the retained introns from the endogenous locus, the sequence for primer 27 was not present (Figure 4.8C). Presumably for the transfection with *aro:loxP::aro[G2A]*, a homology region further upstream might have been used, so that primer 28 was not able to bind. The stretch of homology needed to be between the cut site of the guide and the lethal sequence of the loxP directly upstream of the ATG in order for repair of the DSB to occur without integrating the first loxP. Since guide 1 starts with the ATG just after the lethal loxP, it was virtually impossible for parasites to find a suitable stretch for homologous recombination, resulting in no integration and only WT parasites surviving.

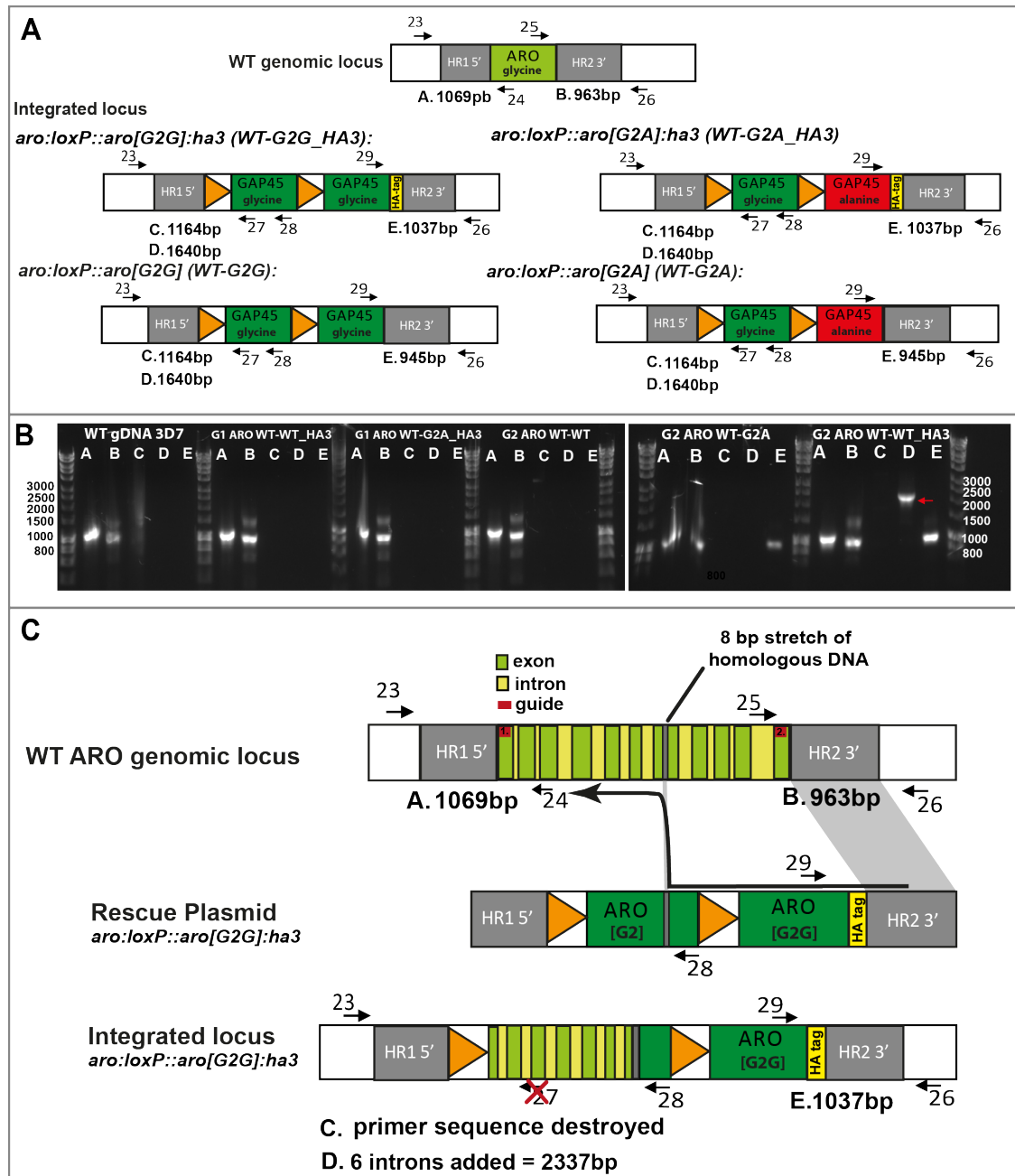


Figure 4.8 Integration PCR of the conditional ARO[G2A] constructs.

A. Schematic of the four ARO constructs showing the primer locations used for the integration PCR. Each construct was used with two guides (G1 and G2). Each primer combination is indicated with a letter (A to E) and length of the PCR fragment (A and B for WT PCT and C to E for integration PCR). B. Agarose gel showing the five successful transfections (three transfections failed during electroporation as parasites recrudesced). Only the last two transfections with Guide 2: *aro::loxP::aro[G2A]* and *aro::loxP::aro[G2G]:ha3* showed integration through at least one positive integration PCR (C to E). C. Graphical representation of the homologous recombination event from the transfection with guide 2 and construct *aro::loxP::aro[G2G]:ha3* explaining the sizes of the PCR fragments in B. Both WT PCRs give a band with the expected size (primer pair A and B). Primer 27 can no longer bind due to the retention of the endogenous sequence generating no PCR product for combination C. PCR reaction D generates a fragment

that is 697 bp bigger due to the retention of the introns in the endogenous region. PCR reaction E produces a product because both primers can bind as the second ARO copy is integrated.

4.2 An alternative strategy: *GAP45*[G2A] complementation in the Δ *GAP45* clone

The integration of sequence to provide a conditional substitution of the N-terminal glycine to an alanine using the DiCre approach failed due to the lethal nature of the presence of loxP directly upstream of the initiator ATG. This was the case for both NMT substrates *GAP45* and *ARO*. Therefore, a different strategy was needed to generate an NMTs[G2A] parasite line. For this approach *GAP45* was selected because a conditional knockout as well as a genetic WT complementation was available thanks to Abigail Perrin (Perrin et al. 2018) (see section 2.2.2.3) (Figure 4.9A). The *gap45:ha3:loxP* has the HA-tagged *gap45* gene flanked by a SERA2-loxPint after the first 49 base pairs (leaving 16 codons non-excised) and a loxP site after the stop codon (Perrin et al. 2018). The genetic complementation integrates a second copy of the *gap45* gene and its own promoter sequence into the *pfs47* locus using the *gap45:ha3:loxP* clone background (Figure 4.9B). The HA-tag of the integrated endogenous *GAP45* will add 4.3 kDa to the molecular weight of *GAP45* which will be visible on a Western blot while the complemented *GAP45* copy, which has no HA-tag will run slightly faster. Both *GAP45* copies are expressed simultaneously from the same promoter at different loci. The genetic complementation *GAP45* construct was used to generate a *GAP45*[G2A] variant. This construct was transfected into the *gap45:ha3:loxP* clone background using the guide sequence previously used for the WT complementation (Perrin et al. 2018) resulting in *gap45:ha3:loxP::comp_gap45*[G2A].

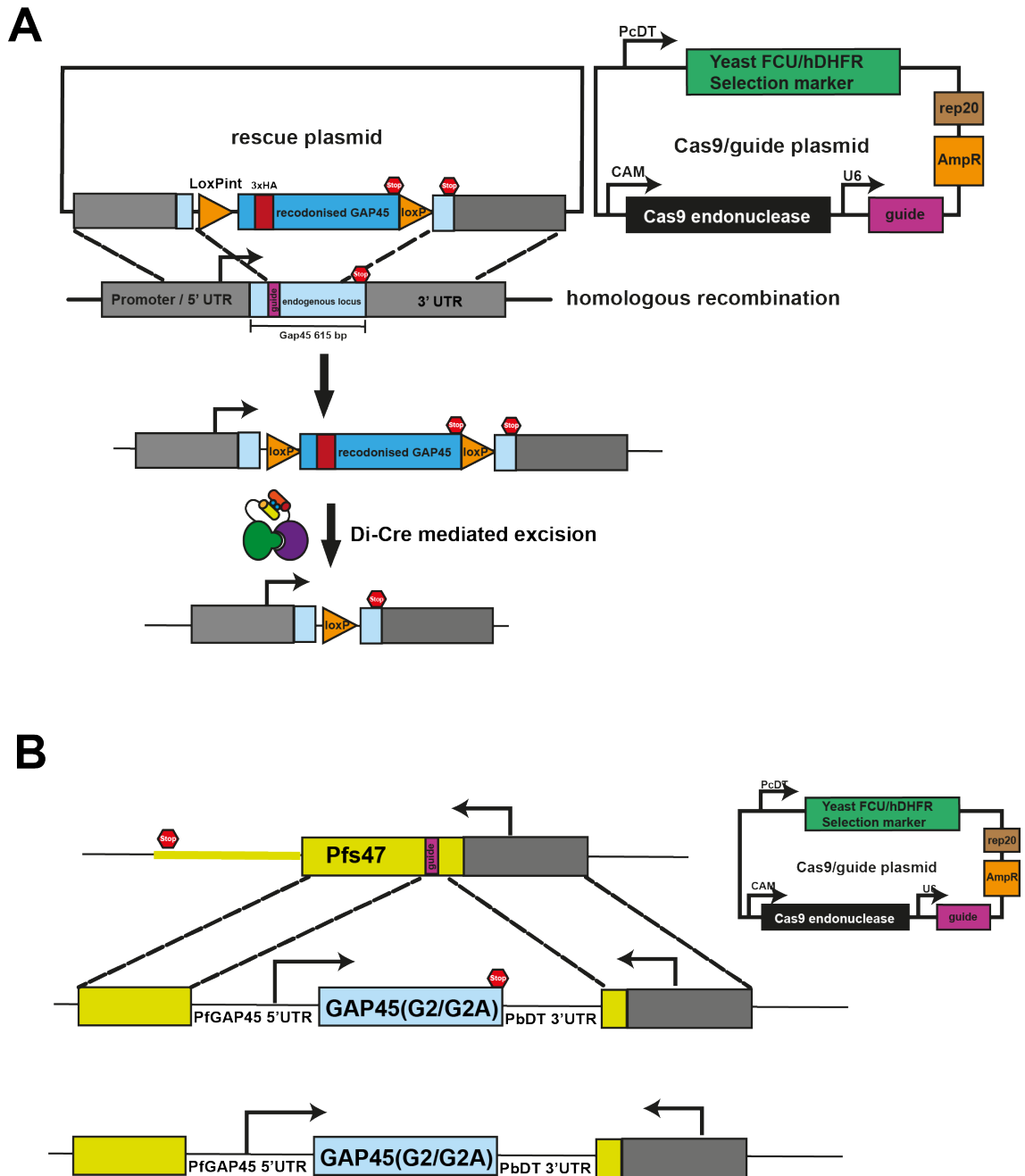


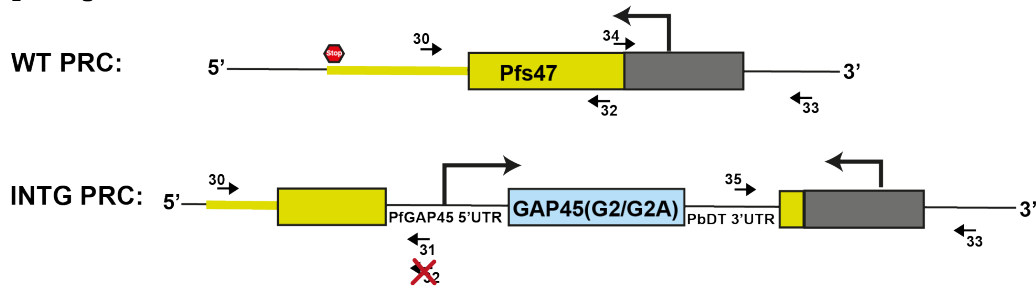
Figure 4.9 The Δ GAP45 and GAP45 complementation construct from Perrin *et al.*, 2018.

A. Schematic representation of the Cas9-based approach for conditional disruption of the *gap45* gene in the DiCre-expressing *P. falciparum* line B11. B. Schematic representation of the genetic complementation strategy used to introduce a second copy of the *gap45* gene coding for either an N-terminal glycine (G2G) or alanine (G2A) and its promoter sequence into the *pfs47* locus of a *gap45:ha3:loxP* parasite clone, generating the *gap45:ha3:loxP::comp_gap45* line or the *gap45:ha3:loxP::comp_gap45[G2A]*.

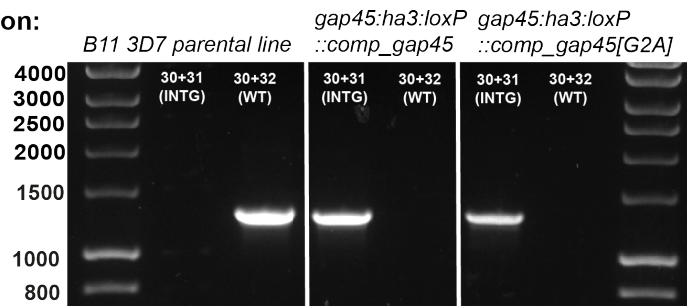
The construct with the WT complementation was transfected at the same time as the control with the construct: *gap45:ha3:loxP::comp_gap45*. Parasites in both

transfections appeared after 22 days and a PCR (Figure 4.10A) showed integration in both lines and no detectable presence of WT parasites (Figure 4.10B). The *gap45-ha3::loxP::comp_gap45[G2A]* line was treated with 10 μ M 5-fluorocytosine (Ancotil) for one week and cloned by limiting dilution, then integration was checked again for each clone (Figure 4.10C).

A Integration PCR at the 5' UTR and 3' UTR



B Uncloned pulation:



C Selected Clones:

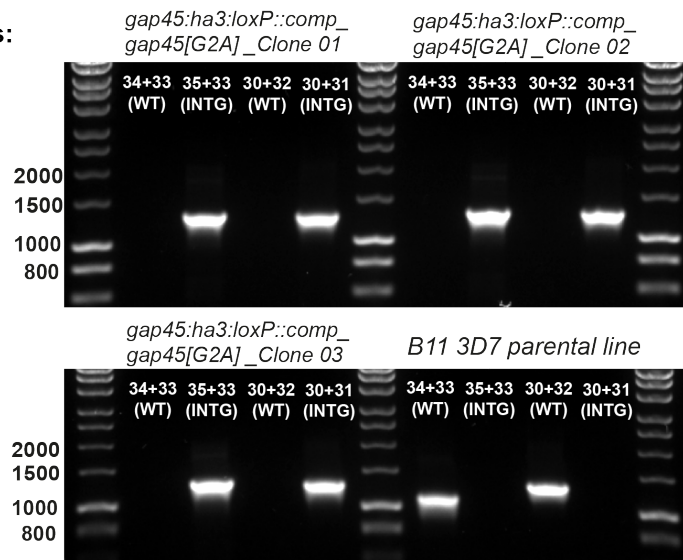


Figure 4.10 Integration PCR before and after cloning of the *gap45:ha3:loxP::comp_gap45[G2A]* parasite line.

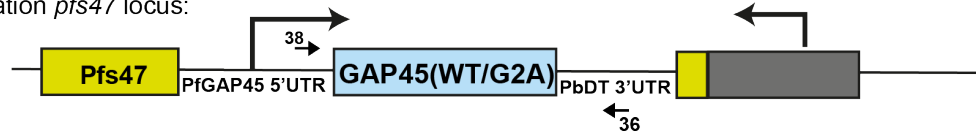
A. Schematic representation of the genetic complementation indicating the oligos used for the integration PCR at the 5' and 3' UTR. B. Integration PCR of the *gap45:ha3:loxP::comp_gap45* and *gap45:ha3:loxP::comp_gap45[G2A]* parasite line showing 5' and 3' UTR integration. The B11 3D7 line was used as a control for the WT

PCR reaction. C. Integration PCR of three selected *gap45:ha3:loxP::comp_gap45[G2A]* clones. The B11 3D7 line was used as a control for the WT PCR reaction.

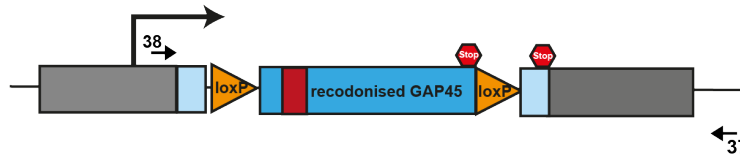
Abigail Perrin provided a *gap45:ha3:loxP::comp_gap45* clone which aided further analysis of the *gap45:ha3:loxP::comp_gap45[G2A]* line. Rapamycin treatment of synchronized *gap45:ha3:loxP*, *gap45:ha3:loxP::comp_gap45*, and two *gap45:ha3:loxP::comp_gap45[G2A]* clones induced Cre recombinase activity and resulted in the anticipated excision of the *gap45:ha3:loxP* gene shown by PCR with primer 38 and 37 (Figure 4.11A, B). The *gap45* and *gap45[G2A]* complementing copies in the Pfs47 locus were not affected by rapamycin treatment (Figure 4.11A,C).

A Excision PCR

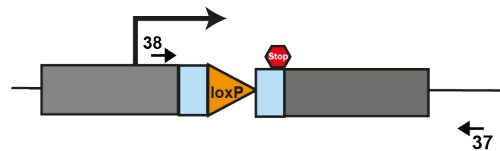
complementation *pfs47* locus:



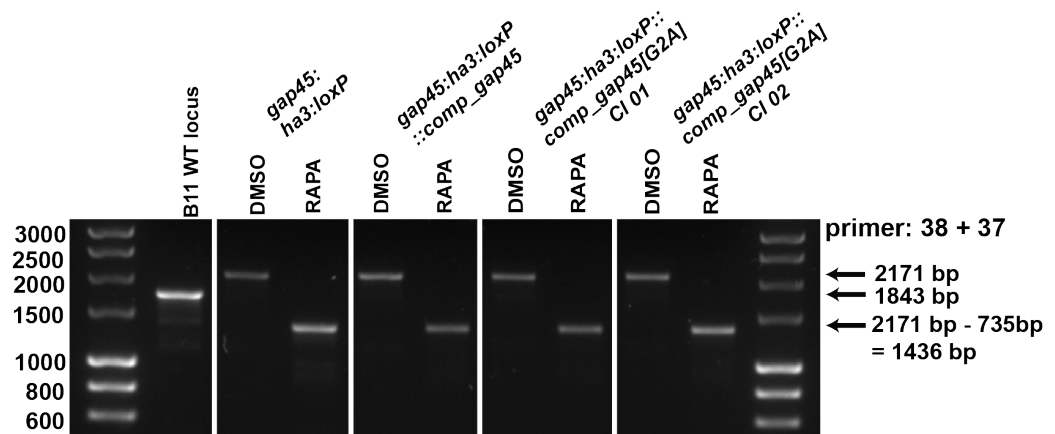
gap45 locus:



excised *gap45* locus:



B



C

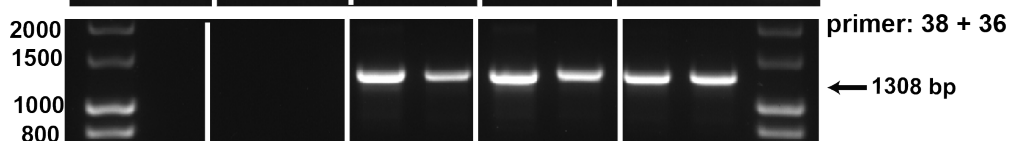


Figure 4.11 Excision PCR with *gap45:ha3:loxP::comp_gap45[G2A]*, *gap45:ha3:loxP::comp_gap45* and *gap45:ha3:loxP* clones.

A. Schematic representation of the genetic complementation indicating oligos used for the excision PCR and oligos used to check presence of complemented construct after rapamycin treatment. B. Excision PCR after rapamycin treatment indicating complete excision in all four clones. C. The complemented *gap45* and *gap45[G2A]* gene is not affected by rapamycin treatment.

In lines treated with rapamycin in cycle 0, GAP45 was completely abolished in the schizont stage by Western blotting in the *gap45:ha3:loxP* line, while both *gap45:ha3:loxP::comp_gap45[G2A]* clones and the *gap45:ha3:loxP::comp_gap45* had the same level of protein compared to DMSO control (Figure 4.12A), showing normal levels of expression of GAP45 in *gap45:ha3:loxP::comp_gap45[G2A]* parasites. This indicates that in both *gap45:ha3:loxP::comp_gap45* and *gap45:ha3:loxP::comp_gap45[G2A]*, GAP45 expressed from the Pfs47 locus is able to complement the excision of GAP45 from its endogenous locus. Morphologically, *gap45:ha3:loxP::comp_gap45[G2A]* develop normally in cycle 0 to schizont stage similar to *gap45:ha3:loxP* and *gap45:ha3:loxP::comp_gap45* confirming the previous observation that GAP45 is not essential for schizont development (Perrin et al. 2018) nor is its N-terminal glycine (Figure 4.12B). However, a longer term viability study over two cycles after rapamycin and DMSO treatment at cycle 0 showed that *gap45:ha3:loxP::comp_gap45[G2A]* was not able to complement the *gap45:ha3:loxP* invasion defect. Rapamycin treated *gap45:ha3:loxP::comp_gap45[G2A]* and *gap45:ha3:loxP* parasites did not proliferate in cell culture (Figure 4.12C). The previously published GAP45 complementation in the *pfs47* locus by *gap45:ha3:loxP::Comp_gap45* was confirmed, and the parasites continued to replicate indicating efficient genetic complementation (Figure 4.12C). This result indicates that the N-terminal glycine and hence myristoylation of GAP45 is indispensable for long-term survival of asexual blood-stage parasites.

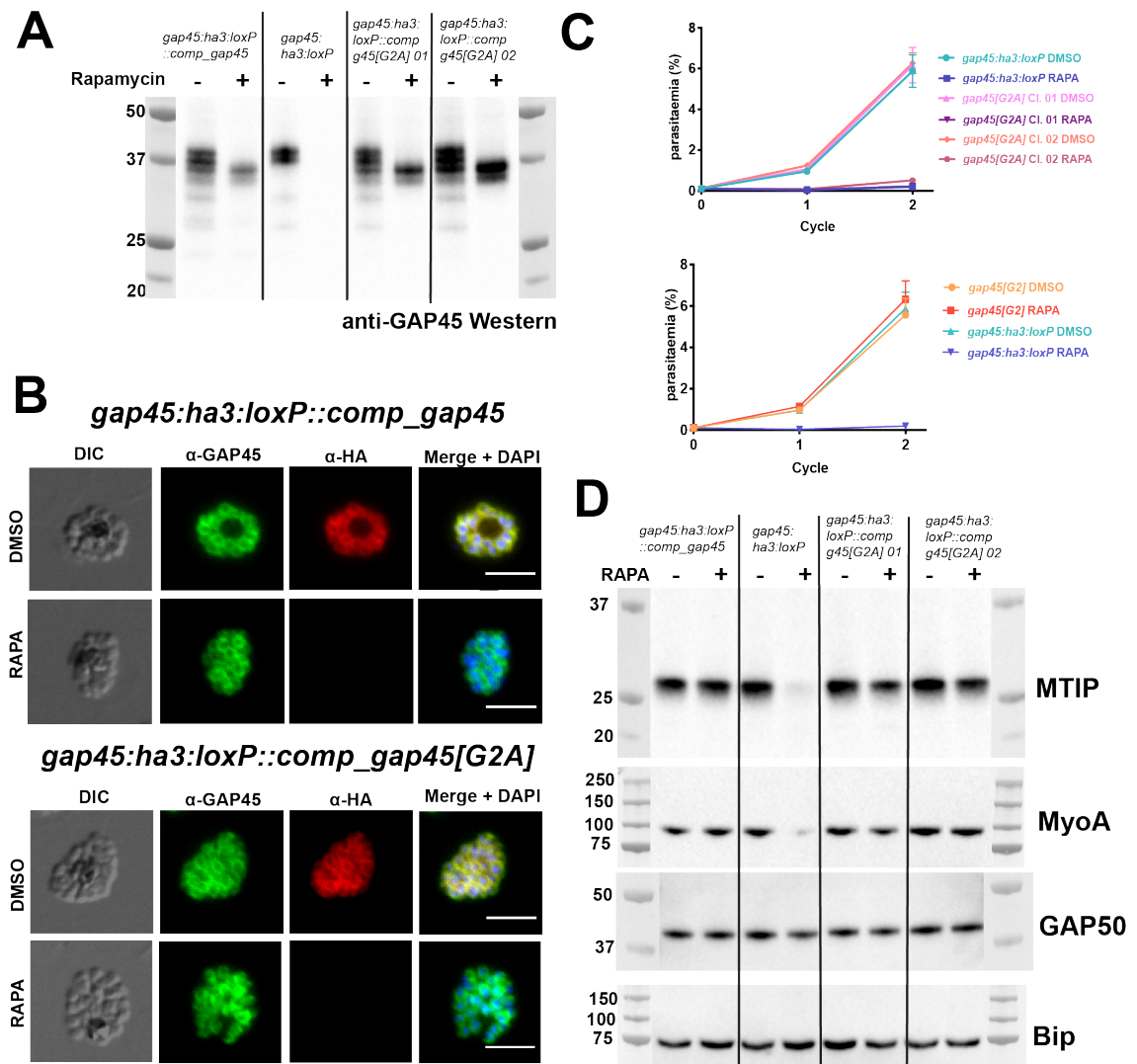


Figure 4.12 GAP45[G2A] shows the same localisation as GAP45, and abundance of other IMC proteins is unchanged.

A. Western blots showing successful rapamycin-inducible ablation of *gap45:ha3:loxP* expression. Note that HA3-tagged GAP45 adds additional mass to the protein resulting in a slower progression through the gel. In both GAP45[G2A] clones the endogenous GAP45-HA3 is deleted but the complemented GAP45[G2A] is still expressed after rapamycin treatment. B. IFA showing the subcellular localization of GAP45-HA3 to the periphery of intracellular merozoites in DMSO treated *gap45:ha3:loxP::comp_gap45* parasite clone and the loss of GAP45-HA3 upon rapamycin treatment. The same can be observed in the *gap45:ha3:loxP::comp_gap45[G2A]* clone. Scale bar, 5 μ m. C. Growth curves showing replication of the *gap45:ha3:loxP* parasite line following rapamycin or DMSO treatment. Rapamycin induced excision of the *gap45:ha3:loxP* locus produced parasites that were unable to replicate *in vitro* which can be complemented by the *gap45:ha3:loxP::comp_gap45* (*gap45[G2G]*) but not by either of the two *gap45:ha3:loxP::comp_gap45[G2A]* clones (*gap45[G2A]* Cl. 01 and Cl. 02). Means from three replicates plotted. Error bars show standard deviations. D. Western blots showing the decreased overall abundance of MTIP and MyoA proteins in the *gap45:ha3:loxP* line after rapamycin treatment, but their presence complementing with *gap45:ha3:loxP::comp_gap45[G2A]* in both clones.

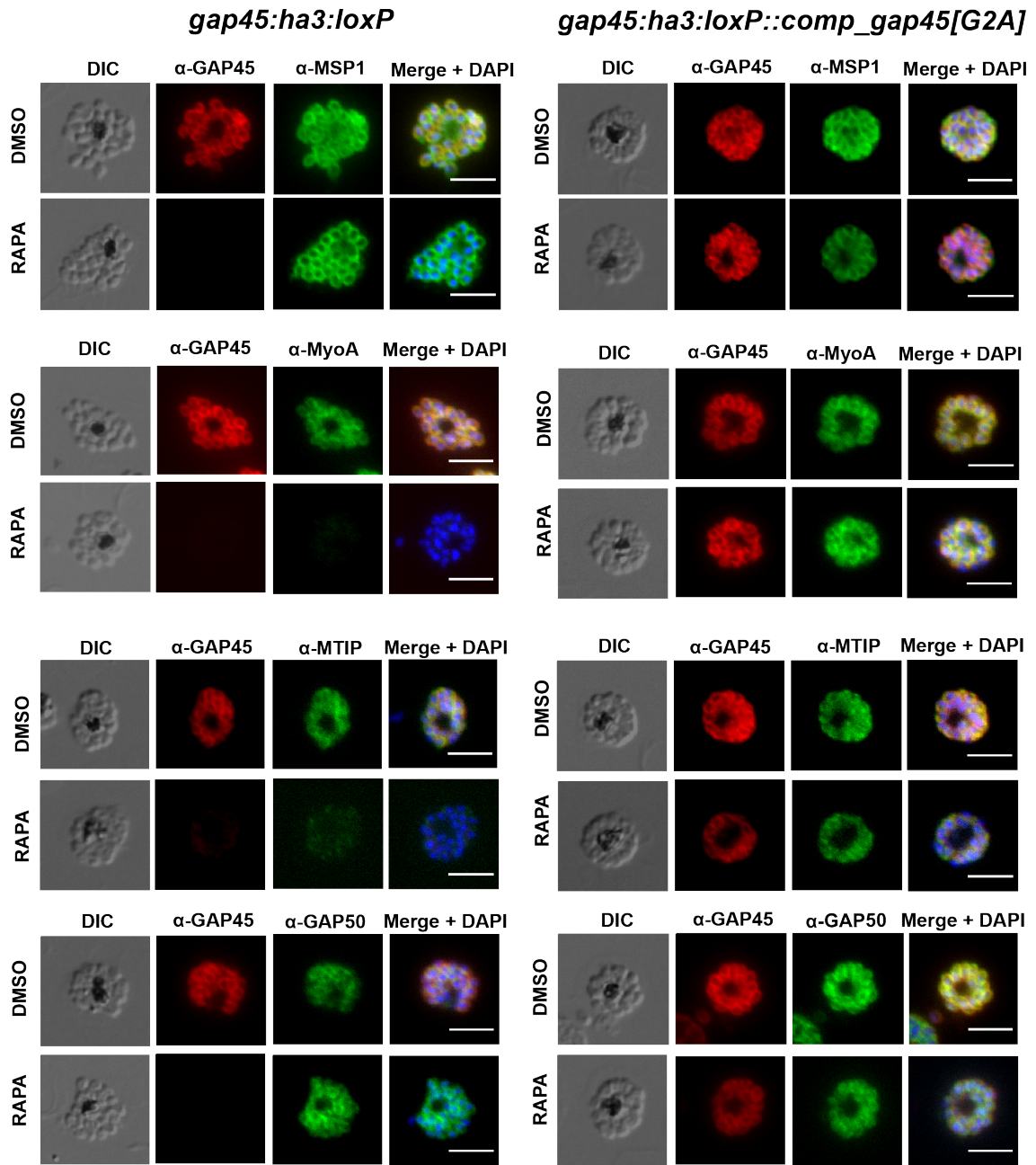


Figure 4.13 GAP45[G2A] parasites have a correctly localised glideosome.

GAP45[G2A] parasites show no defects in expression of the glideosome components MyoA and MTIP and which localised correctly at the periphery of merozoites. IFA showing the subcellular localization of GAP45-HA3, MyoA, MTIP and GAP50 in segmented schizonts of Δ GAP45 (*gap45:ha3:loxP*) and GAP45[G2A] (*gap45:ha3:loxP::comp_gap45[G2A]*) in rapamycin and mock-treated (DMSO) parasites. Loss of GAP45 resulted in loss of detection of MTIP and MyoA at the IMC upon rapamycin treatment, while GAP45[G2A] is still able to recruit MTIP and MyoA. GAP50 and MSP1 staining is unchanged in both lines after rapamycin treatment. Scale bars, 5 μ m.

Comparison with the glideosome assembly in *T. gondii* (see section 1.3) has resulted in a model in *P. falciparum* in which the C-terminal domain of GAP45 recruits the

PfMTIP/PfMyoA complex and binds to the IMC via GAP50 (Frénal et al. 2010). As previously published, in *gap45:ha3:loxP* parasites, MTIP and MyoA are not expressed at WT levels and are not recruited by GAP45 to the IMC as assessed by western blotting and indirect IFA after rapamycin treatment (Perrin et al. 2018). On the other hand, GAP50 was detected apparently at WT levels by western blot and IFA and remained localised at the periphery of intracellular *gap45:ha3:loxP* merozoites. However, electron microscopy showed that IMC formation was not impaired and it was concluded that GAP45 is essential for correct motor complex assembly but not for maintaining the structural integrity of the IMC (Perrin et al. 2018). In *gap45:ha3:loxP::comp_gap45[G2A]*, MTIP and MyoA levels were detected at apparently WT levels on western blots and in the correct location at the IMC by IFA (Figure 4.12D and Figure 4.13). In *gap45:ha3:loxP*, both MTIP and MyoA signals were reduced below a detectable level after rapamycin treatment which is in line with the western blot detection as well as what was reported previously (Figure 4.12D) (Perrin et al. 2018). MSP1 and GAP50 were both correctly located in *gap45:ha3:loxP::comp_gap45[G2A]*, which was expected as rapamycin treated *gap45:ha3:loxP* parasites did not show any mislocalisation of GAP50 and MSP1 (Perrin et al. 2018) (Figure 4.13). Taken together, these findings show that the GAP45 N-terminal glycine is not involved in recruiting MyoA and MTIP to the IMC, nor is the N-terminal glycine essential for IMC localisation. However the growth assay over two generations indicates that there is a severe growth defect of *gap45:ha3:loxP::comp_gap45[G2A]* (Figure 4.12C).

Building on the previous work showing that parasites lacking GAP45 are able egress but not invade, it was investigated if *gap45:ha3:loxP::comp_gap45[G2A]* parasites after rapamycin treatment showed signs of egress but lack the ability to invade new RBCs after rapamycin treatment. Giemsa-stained thin blood smears of rapamycin treated *gap45:ha3:loxP::comp_gap45[G2A]* parasites 48h post invasion showed increased numbers of merozoites but lack any ring-stage parasites compared to the DMSO-treated control (Figure 4.14A). This is similar to what was observed with *gap45:ha3:loxP* parasites. An invasion assay used one *gap45:ha3:loxP::comp_gap45[G2A]* clone, the *gap45:ha3:loxP* clone as well as the *gap45:ha3:loxP::comp_gap45* clone with parasites either treated with DMSO or rapamycin. The assay showed a significantly reduced invasion rate (parasitaemia

cycle 1 / parasitaemia cycle 0) (Welch's unpaired two tailed $p < 0.0001$ for *gap45:ha3:lox* and $p = 0.001$ for *gap45:ha3:loxP::comp_gap45[G2A]*) compared to *gap45:ha3:loxP::comp_gap45* parasites which are able to invade new RBCs after rapamycin treatment (Figure 4.14B). Both *gap45:ha3:loxP::comp_gap45[G2A]* parasites as well as the *gap45:ha3:loxP* show significantly fewer newly formed rings after 24h into the first cycle after rapamycin treatment (Welch's unpaired two tailed t-test $p < 0.0002$ for *gap45:ha3:loxP* and $p = 0.0042$ for *gap45:ha3:loxP::comp_gap45[G2A]*) while the schizont population decreases compared to time points 0 and 4 h PI, consistent with egress occurring normally (Figure 4.14C).

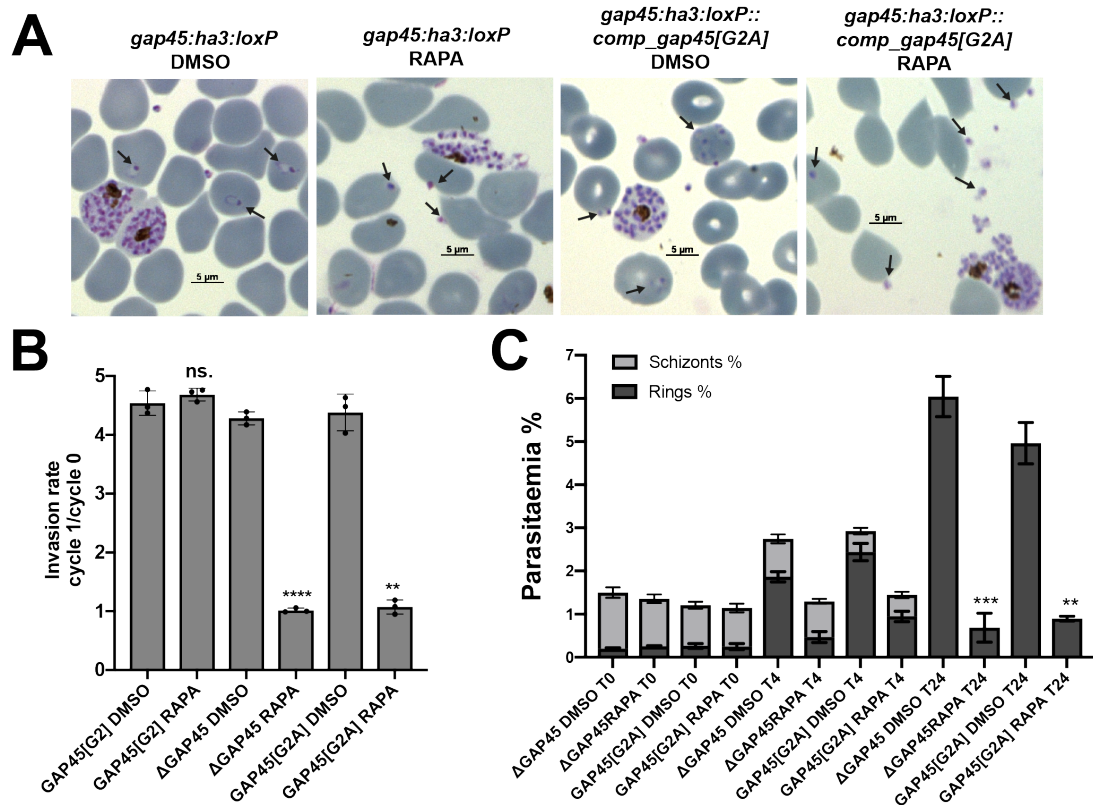


Figure 4.14 GAP45[G2A] parasites show defect in invasion with no block in egress.

A. Giemsa-stained thin blood smears of *gap45:ha3:loxP::comp_gap45[G2A]* and *gap45:ha3:loxP* clones before and after rapamycin treatment showing no newly invaded rings after rapamycin treatment in both lines, while invasion occurs in the DMSO control (black arrows). Despite no invasion occurring after rapamycin there is an abundance of free merozoites visible (black arrows) indicating egress occurred. B. Invasion assay of *gap45:ha3:loxP::comp_gap45[G2A]* (*GAP45[G2A]*), *gap45:ha3:loxP* (Δ *GAP45*), and *gap45:ha3:loxP::comp* (*GAP45[G2G]*) showing the invasion rate (cycle 1 / cycle 0) before and after rapamycin treatment ($n = 3$, Welch's unpaired two tailed $p < 0.0001$ for KO and $p = 0.001$ for G2A); error bars show standard deviation. C. Percentage parasitaemia of schizonts and rings in *gap45:ha3:loxP* (Δ *GAP45*) and *gap45:ha3:loxP::comp* (*GAP45[G2G]*) parasites before and after rapamycin treatment at

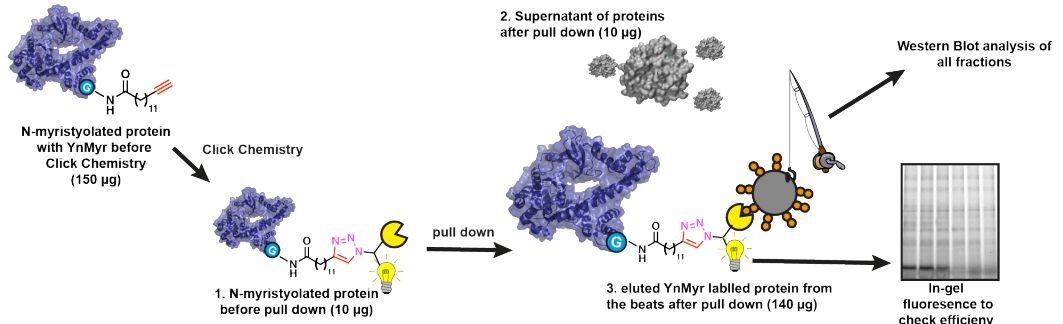
0, 4, and 24 h PI (n = 3, Welch's unpaired two tailed t-test $p < 0.0002$ for KO and $p = 0.0042$ for G2A) error bars show standard deviation.

4.3 GAP45[G2A] is not myristoylated but still palmitoylated

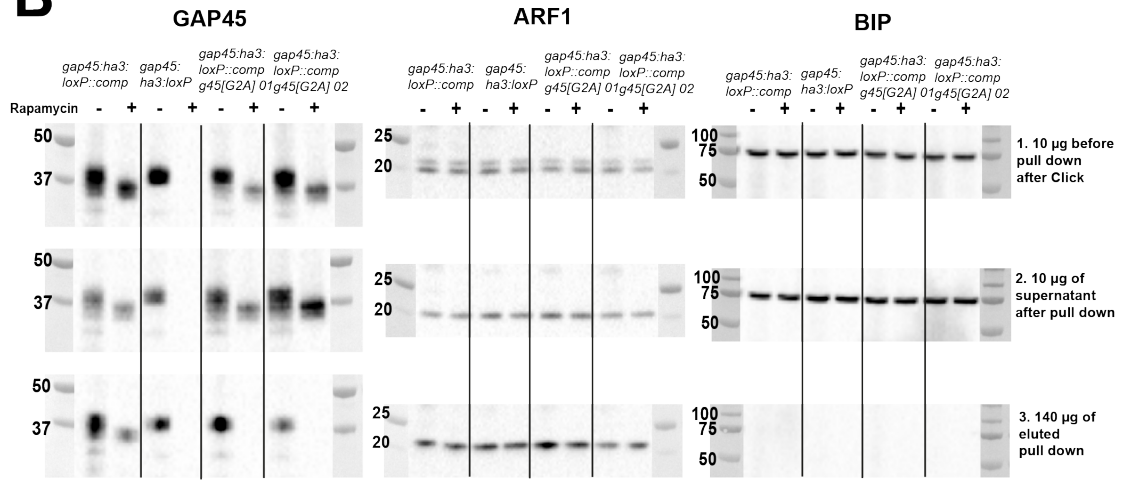
To demonstrate the lack of myristoylation the two *gap45:ha3:loxP::comp_gap45[G2A]* parasite clones were labelled with YnMyr (YnC12) after rapamycin treatment in cycle 0. The *gap45:ha3:loxP::comp_gap45* and *gap45:ha3:loxP* clones served as controls for successful excision of endogenous GAP45 after rapamycin treatment and successful complementation of the myristoylation site. Parasites were labelled with YnMyr (YnC12) at 34 h PI and harvested at the schizont stage (48 h PI) (see section 2.2.4.1). Proteins were extracted (see section 2.2.4.2) and CuAAC-chemistry (see section 2.2.4.3) was used to attach the AzRB capture reagent which carries a TAMRA fluorophore and a biotin tag for pull down (see section 2.2.4.8) (Figure 4.15A). The pull down allowed purification of the YnMyr labelled proteins. To determine whether GAP45[G2A] failed to be pulled down due to the lack of myristoylation, proteins were transferred to a nitrocellulose membrane using the iBLOT Transfer system (see section 2.2.3.2) and probed with anti-GAP45 antibodies to detect the presence of GAP45 in the lysate and in the pull down eluate. While it was possible to pull down GAP45 from the DMSO-treated sample of all 4 lines, only the *gap45:ha3:loxP::comp_gap45* was expressing the myristoylated protein after rapamycin treatment (Figure 4.15B). The positive control ARF1, a known NMT substrate (see section 1.5.2), was successfully enriched through the pull down (note that the additional mass of the YnMyr resulted in a mass shift). The negative control Bip, which is not myristoylated, was not pulled down (Figure 4.15B).

Chapter 4. Conditional G2A substitution of NMT substrates

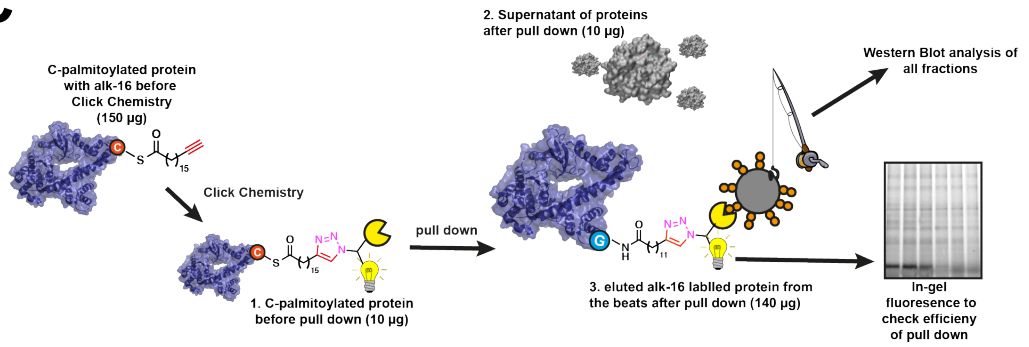
A



B



C



D

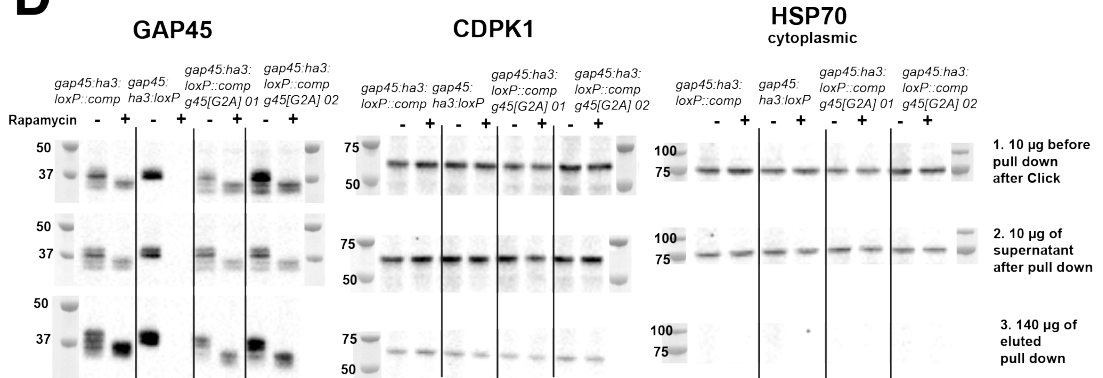


Figure 4.15 GAP45[G2A] is not myristoylated but still palmitoylated.

A. Schematic showing CuAAC (click)-chemistry reaction using the alkyne on the YnMyr and the azide on the capture reagent to attach the fluorophore for in gel fluorescence and a biotin for pull down. The three fractions: before pull down, supernatant and after pull down are numbered. B. Western blot with anti-GAP45, Anti-ARF1, and anti-BIP with lysates from the three fractions in A. After rapamycin treatment only the *gap45:ha3:loxP::comp_gap45* clone contained GAP45 that could be pulled down. All four other clones showed no signal after rapamycin treatment with the anti-GAP45. The NMT substrate ARF1 was used as a positive control and was successfully pulled down from all four clones, while the negative control BIP was not pulled down as it is not myristoylated. C. Schematic showing CuAAC-chemistry reaction using the alkyne on the YnPal and the azide on the capture reagent to attach the fluorophore for in gel fluorescence and a biotin for pull down. The three fractions: before pull down, supernatant and after pull down are numbered. D. Western blot with anti-GAP45, anti-CDPK1, and anti-HSP70. All clones except *gap45:ha3:loxP* expressed GAP45 after rapamycin treatment and pulled down, indicating normal palmitoylation levels of GAP45[G2A]. CDPK1 was used as a positive control which showed a positive pull down signal for all four clones, while HSP70 was not pulled down as it is not palmitoylated.

As discussed in section 1.5.1, GAP45 has six potential palmitoylation sites: one at the N-terminus (C5), and possibly at least one (up to five) at the C-terminus. Myristoylation by itself is thought to be insufficient to anchor the NMT substrate in the membrane (Peitzsch & McLaughlin 1993). Therefore, a second acylation through palmitoylation is necessary to enhance the interaction. Since myristoylation is predominantly a co-translation process it is believed to precede palmitoylation. Since GAP45[G2A] is not myristoylated it was further investigated to see if palmitoylation is occurring at a normal level. This also links to the question of how GAP45[G2A] is localizing to the IMC membrane, which is indicated in the immunofluorescence images shown earlier (Figure 4.13). For this experiment the same four parasites clones were synchronized, rapamycin treated (see section 2.2.1.6) and labelled with YnPal (YnC16) (see section 2.2.4.1) (Figure 4.15C). The CuAAC-chemistry reaction to attached the AzTB was followed by a pull down on streptavidin coated beads and a western blot analysis. Western blots were probed with anti-GAP45, anti-CDPK1 (CDPK1 contains one palmitoylation site at the N-terminus and serves as a positive control for the pull down), and anti-HSP70 (HSP70 is a cytoplasmic protein that contains no palmitoylation sites and acts as a negative control) (Figure 4.15D). Both *gap45:ha3:loxP::comp_gap45[G2A]* clones and the *gap45:ha3:loxP::comp_gap45* clone showed a similar GAP45 signal before and after rapamycin treatment indicating that GAP45[G2A] is palmitoylated to the same extent as GAP45 (Figure

4.15D). The *gap45:ha3:loxP* clone showed successful excision of GAP45 after rapamycin treatment.

The incorporation of YnPal does not give any indication about the number of palmitoylated cysteines in GAP45[G2A]. As mentioned earlier, it is possible that the palmitoylation at the N-terminus might not occur if the preceding co-translational myristoylation is prevented. In order to address the question of how many of GAP45[G2A] cysteines are palmitoylated, a previously published method of acyl-PEG exchange (APE) was used (see section 2.2.4.7). With this technique, the protein is first reduced with Tris(2-carboxyethyl)phosphine (TCEP) and free cysteine residues are capped with N-ethylmaleimide (NEM). This is followed by treatment with hydroxylamine (NH₂OH) to cleave any thioester bonds and therefore remove previously incorporated palmitoylation (see section 2.2.4.7). This treatment allows a site-specific alkylation of the now free cysteine residues with methoxy(polyethylene glycol)-maleimide (mPEG-Mal) mass-tag that adds 10-20 kDa depending on the size of the mass tag used. For this study the addition of a 10 kDa mPEG-Mal was used. Depending on the number of palmitoylation sites that the protein has, multiple mPEG-Mal moieties will be added which will give rise to the same number of distinct band shifts on a western blot. Difficulties in adding mPEG-Mal tags can arise when cysteines are in very close proximity, as it is the case for the two potential GAP45 C-terminal palmitoylation sites. GAP45 forms a 37–39 kDa doublet, since different phosphorylated forms of the protein give rise to two distinct bands on a western blot. The HA-tag of the modified *GAP45* copy at its endogenous locus has an additional 4.3k Da, which is lost together with the GAP45 protein after rapamycin treatment. The mPEG-Mal tags should add a multiple of 10 kDa to the mass of the protein depending on a number of palmitoylation sites. For this experiment the *gap45:ha3:loxP::comp_gap45* clone was used as well as a *gap45:ha3:loxP::comp_gap45[G2A]* clone. Both were treated with rapamycin and DMSO as a control. The samples were split before the NH₂OH treatment, to determine the background of mPEG-Mal tagging. From the western blot two clean sites of mPEG-Mal addition are visible indicating two palmitoylated sites in GAP45 (Figure 4.16A). Each fraction was then quantified measuring the band intensity with ImageJ and expressed as a percentage of the whole signal from the first exposure (Figure 4.16B). A longer exposure was necessary to visualize the second band shift,

indicative of addition of mPEG-Mal to a second palmitoylation site. Evidence of a third palmitoylation site was not visible: such a band would have been at a size of 67 kDa (-HA) and 71.3 kDa (+HA). CDPK1 was used as palmitoylation control (Figure 4.16B). As expected one mPEG-Mal shift was visible for CDPK1, which had only a very faint background signal in the - NH₂OH control (Figure 4.16B).

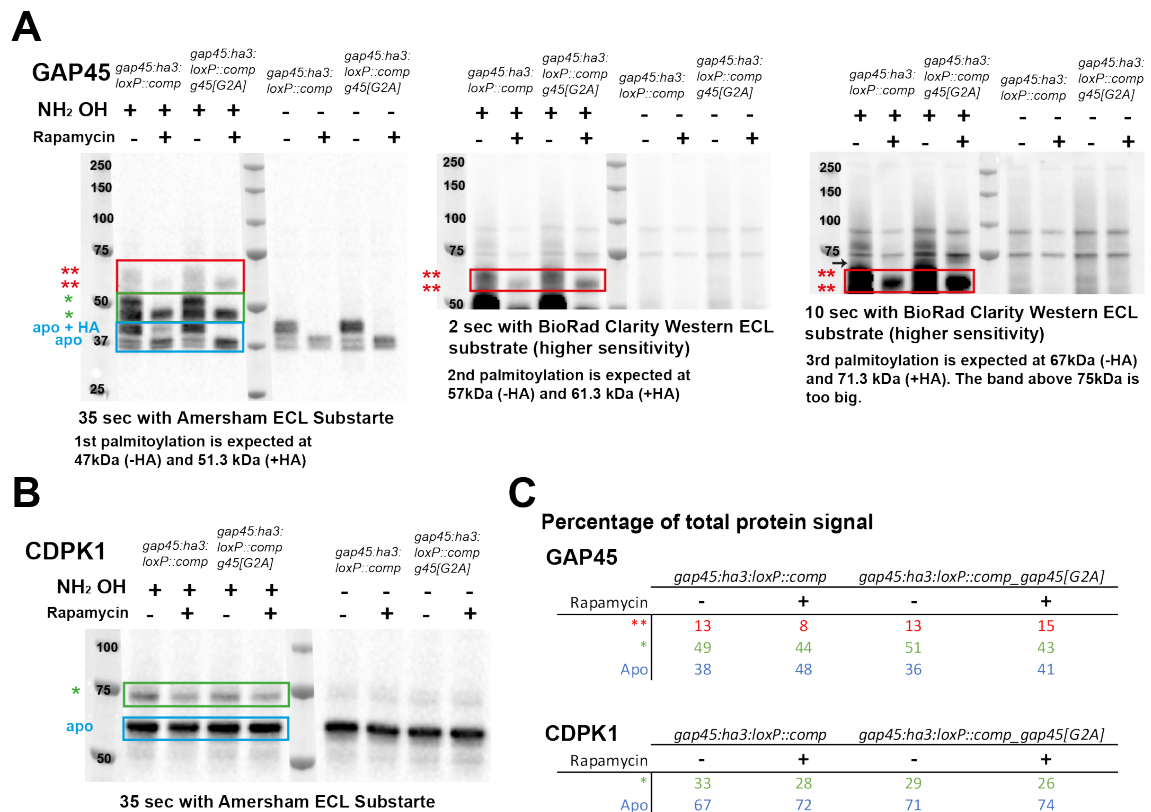


Figure 4.16 Acyl-PEG exchange (APE) reveals site-specific S-fatty acylation of GAP45[G2A] at the same level as that of GAP45.

A. *gap45:ha3:loxP::comp_gap45* and *gap45:ha3:loxP::comp_gap45[G2A]* were treated with rapamycin and DMSO and lysed at 48 h PI. Lysates were subjected to APE, separated by SDS/PAGE, and analysed by Western blot. Notice the shift of apo to apo+HA in blue which indicates the mass shift of the HA-tag in the endogenous locus while the complementation in the *pfs47* locus does not have an HA tag. The number of PEGylation events are indicated by asterisks (*). A third palmitoylation mPEG-mal mass shift for a third palmitoylation of GAP45, would be expected at 67/71 kDa (+/- HA-tag, black arrow in right gel) but there are no additional bands visible at that molecular weight. The western blot with anti-GAP45 is presented after three different exposures, as labelled below the gel. B. CDPK1 was used a positive control. It has one palmitoylation site which is visualised by the one PEGylation event indicated by asterisks (*). C. Semi-quantitative measurement of anti-GAP45 (left gel) and CDPK1 signal indicating the fraction of unmodified versus S-fatty acylated proteins.

4.4 Discussion

In order to study the essentiality of *N*-myristoylation for one particular NMT substrate in isolation, a conditional approach to exchange the N-terminal glycine with an alanine would have been the preferred method. For the second amino acid, following the initiator methionine to be substituted, the full-length coding sequence for the substrate needs to be flanked by two loxP sites in order to be excised using the DiCre approach. In the light of current knowledge, a loxP site has never been successfully placed directly upstream of the ATG start codon in *Plasmodium*. Not much is known about the promoter region in *Plasmodium* making it difficult to predict whether the 34 bp loxP sequence will interfere with transcription. The promoter located 20-3000 base pairs upstream of the transcription start site (TSS) usually consists of a TATA box bound by the TATA-binding protein (TBP), the initiator sequence, a general transcription factor such as TFIIB binding the BRE and a downstream core promoter element (DPE) (Gomez et al. 2010). Together these components will form the transcription preinitiation complex (PIC) recruiting the RNA polymerase II to the promoter. Not many of the general transcription factors associated with RNA polymerase II known in humans have been predicted in *Plasmodium* (Callebaut et al. 2005) and there is only little evidence for the specific binding sequences of the components. In a more recent micrococcal nuclease (MNase) experiment, sequences were protected against processing by MNase digestion when bound by chromatin. Accessible TSS in those nucleosome-depleted regions (NDR) were identified to be located less than 1 kb upstream of the start codon in 81% of the analysed cases and less than 0.5 kb in 50% of the cases (Kensche et al. 2016). A specific combination of polyA and polyT tracks has been found to be a common feature for TSS as well as there being a high abundance of TATA-box-like sequences. But with the average genome-wide AT content being 81% and reaching 90-95% in some regions, it is difficult to detect the specific sequences (Kensche et al. 2016). In *Toxoplasma*, a loxP site has been placed successfully directly upstream of the ATG start codon (Drewry & Sibley 2015) as well as in other organisms such as mice (Weissman & Pan 2015) and lentivirus (Michel et al. 2010). Other studies in *Toxoplasma* used either 3 bp (Egarter et al. 2014; Andenmatten et al. 2013) or 6 bp (Drewry & Sibley 2015) between the upstream loxP and the start

codon, in all cases without much explanation on how the distance was chosen. In this study the loxP was placed directly upstream of the ATG to conditionally exchange the N-terminal glycine of NMT substrates GAP45 and ARO, to an alanine. Attempts failed for both genes with two independent guides. This is most likely due to the lethal nature of the loxP directly upstream of the start codon which might interfere with transcription initiation by blocking the access for the initiation complex or the RNA polymerase II. Integration of the GAP45/ARO conditional G2A construct occurred through a stretch of 8-12 bp homologous sequence downstream of the first loxP facilitating the integration of the remaining construct but preventing integration of the loxP together with the N-terminal-coding sequence of the gene up to the point of the small homologous stretch of DNA. An alternative strategy would have been to place the loxP at the C-terminus of a gene upstream of GAP45/ARO, if the gene is dispensable from asexual stages as the loxP insertion would disrupt its expression. In the case of GAP45 this would be PF3D7_1222600, an AP2 domain transcription factor AP2-G, dispensable for sexual stages (Zhang et al. 2018), which is 4 kbp away from GAP45 in a head-to-tail orientation. The problem with this strategy is that this >4kbp fragment of the C-terminus of AP2-G, the 5' UTR of *GAP45* including the promoter as well as GAP45 itself might form a circular DNA plasmid after DiCre mediated excision. The circular plasmid might facilitate expression of GAP45 at least during the first cycle after excision. At least this is true for enzymes expressed from plasmids which are sufficient to sustain a wild type phenotype due to their high catalytic efficiency (Méthot et al. 2007; Méthot et al. 2008). A recent knock out of *SERA6* with its promoter, by James Thomas in Mike Blackman's lab, also showed that the excised circular DNA plasmids facilitated expression of *SERA6* to an extent that no phenotype was observed until 96 hours post invasion (J. A. Thomas et al. 2018). The plasmid of which there is most likely just one copy will only be present in one daughter merozoite as it presumably is not replicated during schizogony and will only be present in a small population in the following cycle or lost due to instability (more details in PhD Thesis of James Thomas "A protease cascade regulates egress of *Plasmodium falciparum* from the human erythrocyte.").

Because of this potential concern a different strategy was followed up: to complement the recently published Δ *GAP45* (Perrin et al. 2018) with a *GAP45*[G2A] variant. Δ *GAP45* parasites are still able to egress which showed that the actomyosin

motor is not involved in egress. They were however not able to invade new RBCs and there were no signs of RBC surface deformations in the absence of an intact glideosome (Perrin et al. 2018). Some infected RBCs, however, showed signs of echinocytosis. This dysregulation of the RBC membrane phospholipid bilayer is dependent on rhoptry discharge but not on an intact glideosome. A growth and invasion assay showed that the same invasion defect is true for GAP45[G2A], indicating that myristoylation is essential for invasion. In contrast to Δ GAP45, GAP45[G2A] localises at the IMC and is able to recruit MyoA and MTIP. Therefore, parasites possess a fully assembled but yet not functional glideosome. Although difficult to determine through IFA, there is no sign of GAP45[G2A] having no interaction with the PPM through its N-terminus. This indicates that the connection of the N-terminus and the PPM might be unaffected by the G2A mutation because of the functional C-terminus. Pull down experiments with YnMyr and YnPal show that GAP45[G2A] is not myristoylated but still palmitoylated to same extent as wild type GAP45. Both wild type GAP45 and GAP45[G2A] also show the same pattern of palmitoylation following acyl-PEG exchange (APE) with two very clear cysteine palmitoylations. There are six possible palmitoylation sites on cysteines in GAP45. Only one cysteine (C160) was shown to be palmitoylated through mass spectrometry (Jones et al. 2012). C5 at the N-terminus has been predicted to be palmitoylated (Rees-Channer et al. 2006) using the CSS-Palm 4.0 prediction tool (score of 35.96) (Ren et al. 2008) and studied in a GAP45₂₉C5A GFP-tagged episomally expressed mutant showing that C5 palmitoylation contributes to membrane association but is neither necessary nor sufficient (Ezougou et al. 2014). The remaining four cysteines are also potential palmitoylation sites (C176, C178, C189 and C192), which have not been validated experimentally and have significantly lower scores compared to C5 using the CCS-Palm 4.0 (score of 2.3-7.0) (Ren et al. 2008). A C-terminal YFP tagged GAP45 of the last 175 amino acid (not including the first 30 amino acids of the N-terminus), has been shown to localize to the IMC (Ridzuan et al. 2012). This sequence encodes all of the five C-terminal cysteine residues, leaving the question open which of them is essential for the interaction (Ridzuan et al. 2012). Both the N- and C-terminus of GAP45 are strikingly conserved across the Apicomplexa and from cysteine mutant work in *Toxoplasma* it is known that the last two cysteines C230 and C233 (conserved with C189 and C192 in *P. falciparum*) in *Toxoplasma* are essential for anchoring GAP45 to the IMC (Frénal et al. 2010). In a double mutant of C230A

and C233A, IFA images showed that GAP45 still localises to the PPM and positions the motor properly at the pellicle (IMC and PPM) but lost the interaction to the IMC (Frénal et al. 2010). Unpublished work from Tatianna Wai Ying Wong, a student in Tim Gilberger's lab, extends on a publication in 2015, on the role of palmitoylation for protein recruitment to the IMC (Wetzel et al. 2015). She showed that a "palminull" mutant of PfGAP45 where all five C-terminal cysteines were mutated to an alanine, shows localisation to the PPM instead of the IMC (T. W. T. Wong 2015). Furthermore, using individual cysteine to alanine overexpression mutants, Tatianna Wai Ying Wong showed that C1601A, C176A and C178A mutants did not re-localise GAP45 to the PPM away from the IMC, while C198 and C192 were individually essential for correct IMC localisation and showed a PPM localisation upon mutation (T. W. T. Wong 2015). In both studies on PfGAP45 and TgGAP45 it is unclear whether the cysteines are involved in anchoring to the IMC, because of their potential palmitoylation or because they are essential for correct protein folding. For example the coiled-coil domain of residues 14-123 may lead to interaction with GAP50 and/or GAP40 and might require the cysteine residues to facilitate this interaction (T. W. T. Wong 2015; Frénal et al. 2010). Changing the length of the coiled-coil domain and therefore the rigidity of TgGAP45 changes its ability to recruit the motor to the pellicle (Frénal et al. 2010). GAP50 and GAP45 have been found to form a complex in *Toxoplasma* through immunoprecipitation (Gaskins et al. 2004) and later in *P. falciparum* (Yeoman et al. 2011). GAP50 is co-translationally embedded into the ER and transported to the IMC through an N-terminal signal peptide and contains a C-terminal transmembrane domain which acts as a membrane anchor for the glideosome (Yeoman et al. 2011). Another possible interacting partner of GAP45, in addition to MTIP (MLC1 in *Toxoplasma*) and GAP50, is GAP40 which was shown to form a complex with MyoA, MTIP/MLC1, GAP45 and GAP50 in *P. falciparum* (Green et al. 2017) and in *Toxoplasma* (Frénal et al. 2010). GAP40 has nine transmembrane spanning domain and could also serve as an anchor point of the glideosome to the membrane.

It is not clear which of the six cysteines is palmitoylated on GAP45 and GAP45[G2A] in this study. Given the close proximity of C5 to the N-terminal myristoylation, the necessity of a second modification of the protein to complement myristoylation (Peitzsch & McLaughlin 1993), and the high prediction score of C5 to be

palmitoylated (Ren et al. 2008), it is likely that one of the shifts indicates palmitoylation of C5. This would however also mean that C5 can still be palmitoylated in the absence of myristoylation in the GAP45[G2A] mutant. This might be possible but unlikely because of the co-translational nature of myristoylation preceding the post-translational palmitoylation. It is however possible that GAP45[G2A] which is still able to interact with GAP50 and GAP40, and able to recruit MTIP and MYoA, is palmitoylated at C5 at the same time as the second palmitoylation occurs at its C-terminus both independently of myristoylation. The second molecular weight shift observed through APE in this study might be the palmitoylation of C160 which has been shown to palmitoylated by MS (Jones et al. 2012). Because of the large size of the mPEG-Mal added to the cysteine of GAP45 it is also possible that more palmitoylation sites were not able to be detected. In order to show that C5 and C160 are palmitoylated, individual cysteine mutants could be generated and the same APE experiment repeated. No addition of mPEG-Mal to the cysteine mutants would show that they are no longer palmitoylated. Considering the unpublished work of Tatianna Wai Ying Wong, GAP45 is presumably localised at the IMC through C198 and C192 by either palmitoylation or correct folding allowing interaction with GAP50 and/or GAP40 and to the PPM only through the N-terminal C5 palmitoylation, but only when the C-terminus initiates localisation first and allowing palmitoylation of C5 to occur at the PPM in the absence of myristoylation. This is because a C-terminal truncated GAP45₂₉C5A does still associate with the membrane but only in the presence of the N-terminal myristoylation and not in a GAP45₂₉C5A, G2A double mutant (Ezougou et al. 2014). Therefore, the initial interaction with the PPM to localize GAP45 at the interface between IMC and PPM must occur through its C-terminus.

In summary, the attempts to conditionally mutate the codon for the N-terminal glycine to an alanine in *GAP45* and *ARO* have shown that the loxP site is lethal directly upstream of the ATG start codon. Since this conditional G2A mutation approach has proven to be difficult, a complementation approach with a G2A variant of the gene knockout was chosen for GAP45. The complemented GAP45[G2A] mutant is not myristoylated and the phenotype shows that myristoylation is essential for the correct function of the glideosome as GAP45[G2A] parasites are not able to invade. Interestingly, the actomyosin motor complex is still assembled in GAP45[G2A]

parasites, unlike in the $\Delta GAP45$ line and palmitoylation is unaffected. This indicating that myristoylation is important for the function of the motor but not for its assembly.

Chapter 5. A novel antimalarial resistance mechanism leads to structure-guided identification of resistance-breaking *Plasmodium* *N*-myristoyltransferase inhibitors

Selection of drug resistance can be exploited in experimental research to identify and validate novel drug targets. This can help in understanding the molecular mechanism of inhibition as well as guide target drug discovery by improving inhibitor binding, specificity and selectivity. It can also be used to show on-target specificity in addition to the TC_{50} / EC_{50} ratio (see section 3.1). In this chapter the first enzyme variant, PfNMT[G386E] is reported which gives rise to parasites resistant to NMTi IMP-1002. On target specificity is evaluated by introducing the same amino acid substitution through genome editing by CRISPR-Cas9 which gave rise to parasites showing the same resistance to IMP-1002. Experimental characterisation of the impact of the amino acid substitution was done using the CRISPR-Cas9 edited PfNMT[G386E] parasite line as well as a biochemically purified PvNMT[G386E] enzyme. This also allowed the identification of a compound series that can overcome parasite resistance. Finally, this chapter shows the binding mode to the wild type PvNMT enzyme (PvNMT[G386]) as well as to PvNMT[G386E] through X-ray crystal structures with the compound that results in resistance (IMP-1002) as well as with selected compounds from the series that can overcome it. Overall this chapter reports the first recorded mechanism of NMTi resistance during the drug discovery process and uses a structure-guided approach to find compounds to overcome it. This whole chapter has been published in Schlott et al., and adapted for this thesis (Schlott et al. 2019).

5.1 A single amino acid substitution in the *P. falciparum* *N*-myristoyltransferase active site leads to parasite resistance to the NMT inhibitor IMP-1002.

As described in section 3.1, IMP-1002 (Figure 1.7) (Schlott et al. 2019) has an increased potency in parasite killing compared to previously published NMTi (Wright et al. 2014) but has the same phenotype in blocking schizonts at morphologically

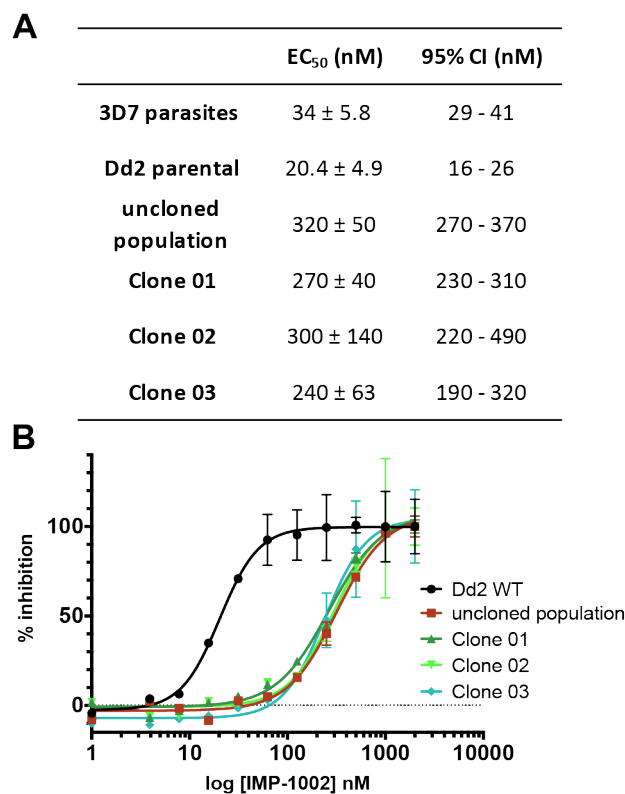


Figure 5.1 IMP-1002 inhibition of parental and resistant parasite lines.

A. Inhibition of *P. falciparum* 3D7 and Dd2 parental parasites used to raise the resistant clones 01, 02, 03 and the uncloned population, showing the respective EC₅₀ and CI value rounded to two significant figures. B. Measurement of the inhibition of growth of the Dd2 parental line, the resistant uncloned population and three IMP-1002-resistant clones to determine the EC₅₀ after 72 hours (n = 3). There is a clear shift of the EC₅₀ for the resistant bulk population and the three parasite clones. Error bars indicate standard deviation of the mean.

distinct “pseudoschizont” stage with 4-6 nuclei, compared to 20 nuclei, when drug treatment is started at ring stage. For drug resistance selection with IMP-1002, the *P. falciparum* Dd2-B2 parasite line (recloned from the Dd2 parental line by limiting dilution) in David Fidock’s lab was used. The EC₅₀ of IMP-1002 was comparable in the Dd2 line and the previously used 3D7 line (Figure 5.1A) (Schlott et al. 2015; Schlott et al. 2019) (examined in MRes thesis). Resistance selection was conducted by Olivia Coburn-Flynn and occurred after 36 days in the presence of 120 nM of IMP-1002 (3.4-times the EC₅₀ in parasite lines) at a starting inoculum of 10⁶ in one out of three replicates. No parasites appeared at a starting inoculum of 10⁷ parasites. From the resistant bulk population, three clones were selected. Resistance to IMP-1002 was confirmed in the bulk population and the three clones with a 14-fold

increase in IMP-1002 EC_{50} (Schlott et al. 2015; Schlott et al. 2019) (examined in MRes thesis) (Figure 5.1A B). Sequencing of the *nmt* gene from genomic DNA of two clones, the resistant bulk population and the Dd2 parental line, revealed the genetic basis for this shift in EC_{50} (Schlott et al. 2015) (examined in MRes thesis). A single non-synonymous point mutation (G1544A) leading to an amino acid substitution from glycine to glutamic acid (G386E) in the active site of the NMT protein was present in the bulk population and each individual clone (Schlott et al. 2015; Schlott et al. 2019).

5.2 CRISPR-Cas gene editing demonstrates that PfNMT[G386E] is sufficient to mediate resistance to IMP-1002.

To confirm that the G386E substitution is sufficient for IMP-1002 resistance, the same mutation was introduced into the *nmt* gene in 3D7 parasites using the CRISPR-Cas9 approach (Figure 5.2) (see section 2.2.1.4 and 2.2.2.5). Construct generation is described in section 2.2.2.4. The G1544A mutation as well as additional shield mutations, which introduced unique restriction sites in a PCR product amplified from the integrated parasite population was introduced using two guide sequences in independent transfections (Figure 5.2A). This exchanged a 23 bp (guide 1) and 68 bp (guide 2) sequence of the endogenous gene (Figure 5.2A). Parasites were visible 17 days post-transfection. Genomic DNA was extracted (section 2.2.1.3) and screened for DNA integration through PCR and analytical digest using the unique restriction sites in the integrated sequence (Figure 5.2D, E). Parasites were subsequently treated with 5-fluorocytosine (Ancotil), and cloned by limiting dilution using the plaque assay to confirm single parasites per well (section 2.2.1.4) (J. A. Thomas et al. 2016). Following cloning, four clones were selected for sequencing of the *nmt* gene as well as tested for the absence of Cas9 plasmid / WR resistance. Two clones from the two independent transfections were selected for an SYBR Green-based growth assay (see section 2.2.1.10), revealing a significant 22-fold increase in IMP-1002 EC_{50} over the parental 3D7 line (Figure 5.3) (Unpaired Welch T-test not assuming equal standard deviations, $p = 0.01$). The provided data confirms that the G386E amino acid substitution is responsible for resistance to IMP-1002.

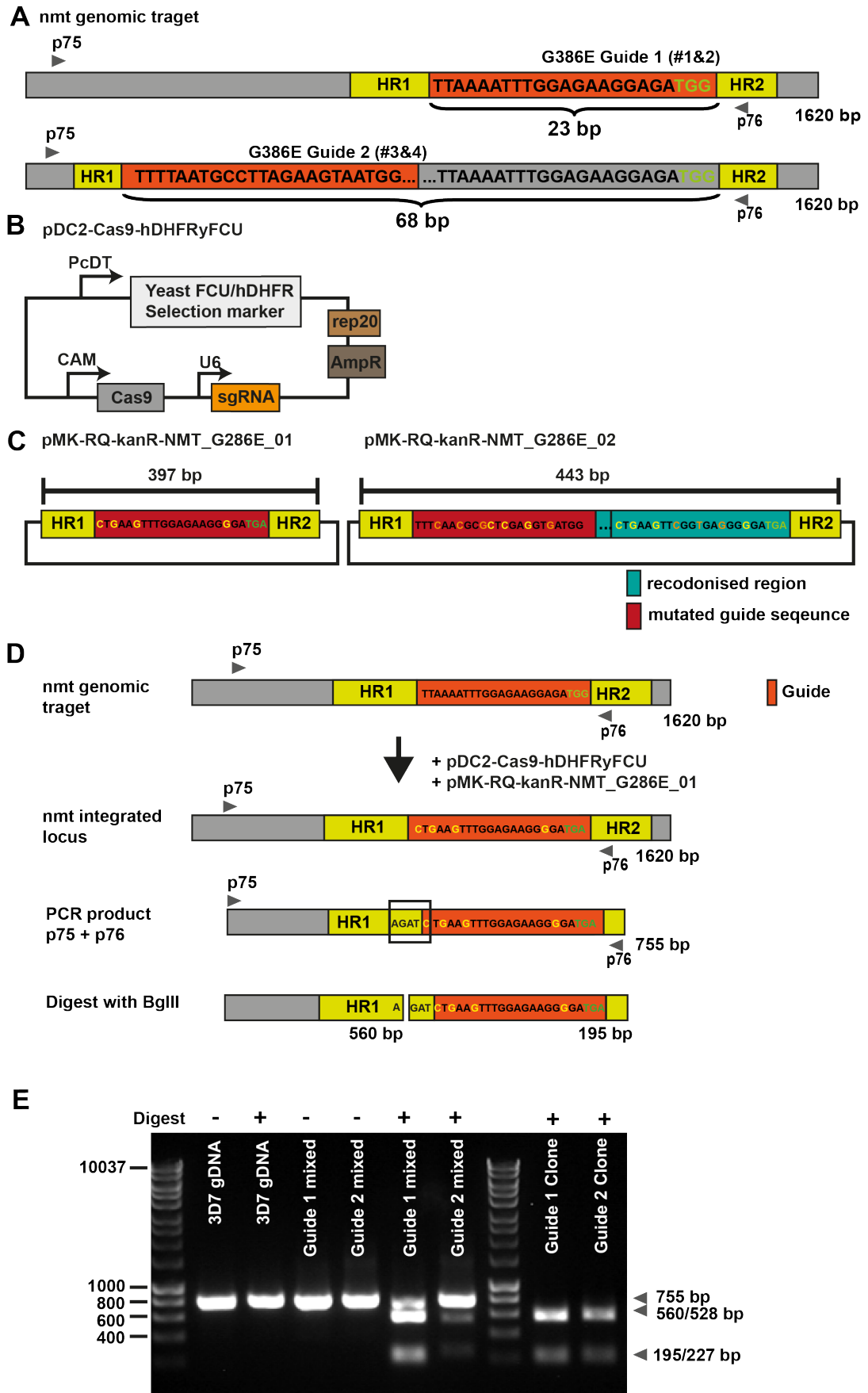
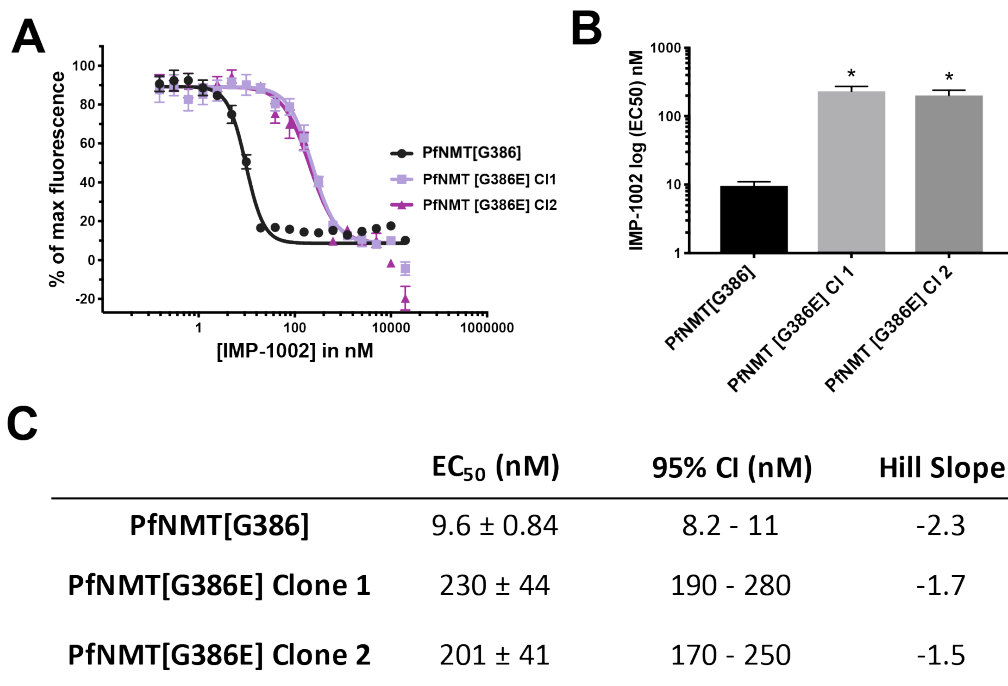


Figure 5.2 Generation of *P. falciparum* parasite lines containing the G386E mutation in the *nmt* gene.

A. Schematic of WT *nmt* ORF highlighting the two guide sequences and sequence before the desired mutation codon TGG highlighted in green (GGA encoding glycine). The PAM sequence of guide 1 was destroyed and mutated to yield the desired G386E mutation (TGG changed to TGA). For Guide 2 that lies upstream of the introduced mutation, a stretch of 45 bp was recodonised. For guide sequences see Guide table (Oligo numbers are given in schematic above sequence and can be referred to in table 2.1) B. Schematic of the Cas9/guide RNA plasmid. C. Schematic of the rescue plasmids with the modified open reading frame (OFR) containing the G386E point mutation (TGA) in green. Homology regions (HRs) on rescue plasmids used for targeting were 200 bp (HR1) and 169/174 bp (HR2) long. The two mutated guide sequences are shown in red. Silent point mutations for the generation of unique restriction sites in the integrated construct are highlighted in yellow. Additional silent mutations are highlighted in orange. d) Screening by PCR and restriction enzyme digest to distinguish WT from integrated parasites. Only integration of G286E_01 is shown. The restriction site is highlighted by a box. For primer sequence see primer table. E. Agarose gel staining for DNA showing restriction digest of DNA amplified from parental line (3D7), mixed population for both transfections and the two clones used for the analysis from two independent transfections.

**Figure 5.3 Growth assay with *P. falciparum* 3D7[G386E].**

A. Growth curve of two PfNMT[G386E] clones and PfNMT[G386] wild type (WT) showing shift in EC₅₀ for both clones from two transfections with independent guides (n = 3). B. EC₅₀ of all three lines plotted in a bar graph. Unpaired Welch T-test not assuming equal standard deviations, p = 0.01. C. Raw data table showing EC₅₀, 95% confidence interval and Hill slope of graph in A. Error bars indicate standard deviation of the mean.

5.3 There is no phenotype associated with PfNMT[G386E] *in vitro*.

The *in vitro* selection of parasites expressing PfNMT[G386E] with IMP-1002 could result in a fitness cost associated with the G386E amino acid change interfering with substrate binding to the PfNMT substrate binding pocket. There are a large number of NMT substrates in *P. falciparum* that must be myristoylated. Therefore, it was examined whether any resultant phenotype could be detected at the level of protein myristoylation, cell cycle progression or long-term growth rate.

To determine parasite growth over several cycles, two PfNMT[G386E] clones as well as 3D7 WT parasites were synchronized and diluted down to a starting parasitaemia of 0.8% in three individual dilutions per parasite line (see section 2.2.1.9). Each individual dilution had three technical replicates throughout the growth assay. The growth rate of the parasites was followed over six cycles. After each replication, the parasitaemia of the WT population (PfNMT[G386]) was determined by Giemsa stain and diluted down to 0.8%. The two PfNMT[G386E] clones were diluted by the same factor. Figure 5.4A shows the relative parasitaemia normalized to the WT population of the three lines over the course of the six generations. A multiple t-test analysis (two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with false-discovery rate = 1 %) showed that there was no significant difference in relative parasitaemia between the three lines.

To investigate potential differences in myristoylation pattern showing substrate engagement, both 3D7 WT parasites and PfNMT[G386E] parasite clones were labelled biosynthetically with the myristic acid analogue YnMyr (YnC12) throughout the complete intraerythrocytic cycle (section 2.2.4.3). Samples were treated with 0.2 M NaOH to remove the base-labile ester-linkage of YnMyr to GPI-anchored proteins (section 2.2.4.4). Both samples before and after NaOH treatment were analysed on an SDS-PAGE Gel and fluorescence intensities of YnMyr labelling were compared to the Coomassie blue stained protein content. There was very little difference in labelling patterns between the three samples, indicating that there is no detectable effect of the G386E substitution on global myristoylation (Figure 5.4B).

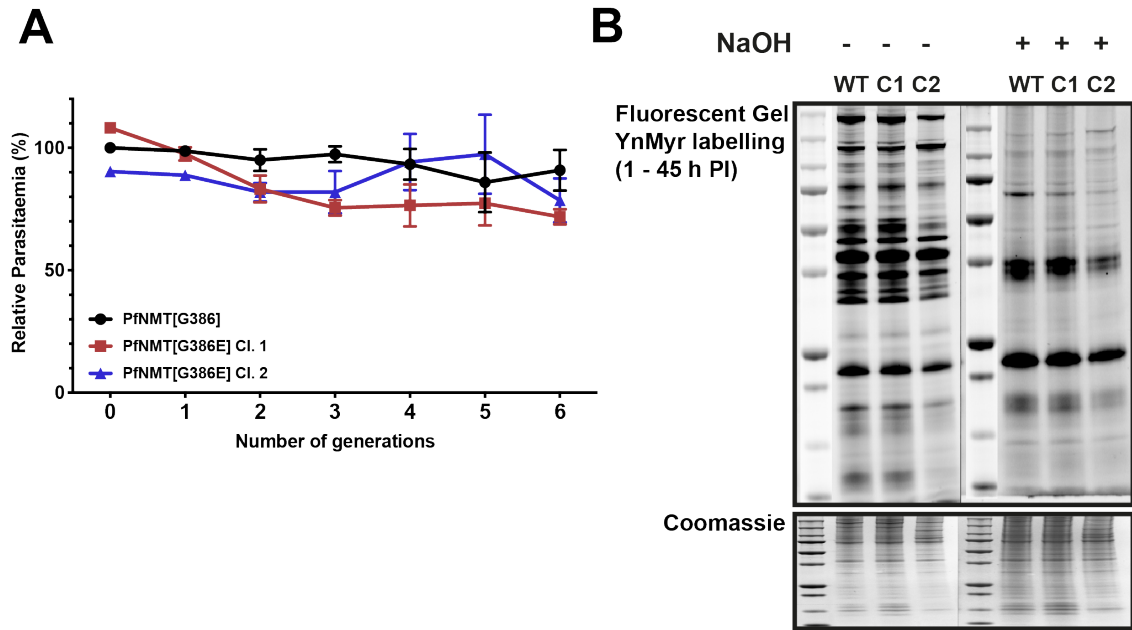


Figure 5.4 *P. falciparum* NMT[G386E] parasites do not show a phenotype and have normal myristoylation levels.

A. Growth assay of *P. falciparum* 3D7 WT parasites and two PfNMT[G386E] clones over six generations. Starting parasitaemia was determined by flow cytometry (Hoechst stain). PfNMT[G386] parasitaemia was determined after each cycle and a dilution factor calculated to give 0.8% parasitaemia. All three lines were diluted using this same factor to avoid cultures overgrowing. Parasitaemias were measured after each cycle using flow cytometry. Total parasitaemia was normalized to the DMSO control replicate with the highest value. N = 3 biological replicates with 3 technical replicas each. Error bars indicate standard deviation of the mean. Multiple t-tests were performed for each generation (two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with false-discovery rate = 1 %). There was no significant difference in relative parasitaemia between the three lines. **B.** YnMyr labelling of PfNMT[G386E] clones (C1 and C2) and PfNMT[G386] (WT) parasites during the entire erythrocytic life cycle. YnMyr labelling was subjected to base (NaOH) treatment to hydrolyse the base-labile incorporation of YnMyr into GPI-anchored proteins. Coomassie stain indicates loading for each lane.

5.4 PfNMT[G386E] parasites resist pharmacological inhibition of myristoylation and parasite development, confirming on-target specificity of IMP-1002.

As discussed in section 3.1, parasites treated from ring stage onwards with IMP-1002 are trapped in a morphologically distinct “pseudoschizont” with 4-7 nuclei while control parasites developing normally to ~20 nuclei at 45 h post-invasion (Wright et al. 2014; Schlott et al. 2015). To further address the on-target specificity of IMP-1002 it was investigated whether PfNMT[G386E] parasites also resist inhibition of *N*-myristoylation preventing formation of the “pseudoschizont” phenotype allowing

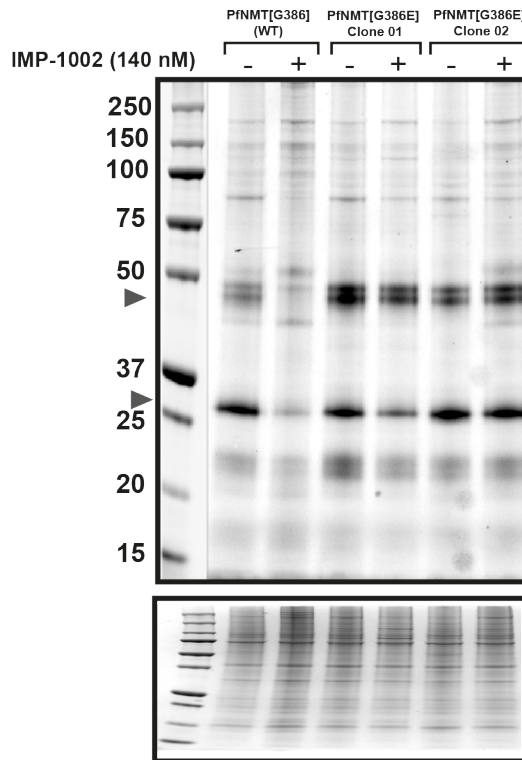


Figure 5.5 IMP-1002 shows on-target specificity through loss of myristoylation signal reversion.

NMT substrates labelled with YnMyr in PfNMT[G386] (WT) and PfNMT[G386E] parasites after treatment with base (NaOH) to hydrolyse the base-labile ester-linkage incorporation of YnMyr into GPI-anchored proteins. While WT parasites show a decreased band intensity in response to the NMTi treatment, PfNMT[G386E] parasite proteins remain unchanged compared to those of the DMSO control. The Coomassie Blue-stained gel shows proportional loading of total protein for each sample.

myristoylation to occur at normal level comparable to 3D7 WT parasites. PfNMT[G386] (WT) and PfNMT[G386E] parasite clones were labelled with YnMyr (YnC12) in the presence or absence of 140 nM IMP-1002 (approximately four-fold EC_{50} for wild type 3D7 parasites) during the period 36-45 h PI, when myristoylation is at its peak (Wright et al. 2014; Schlott et al. 2018). Whilst there was a clear decrease in YnMyr labelling in WT parasites treated with IMP-1002, there was no reduction in labelling of the PfNMT[G386E] parasite clones with the inhibitor present (Figure 5.5).

To determine whether PfNMT[G386E] parasite clones develop normally in the presence of 140 nM IMP1002, synchronized ring stages from WT parasites and a PfNMT[G386E] clones were treated with either 140 nM IMP-1002 or control DMSO for a period of 45 h. The development was followed until just before egress and parasite stages were determined by Hoechst staining and flow

cytometry (see section 2.2.1.8). A sample only containing uninfected RBC was used as a Hoechst negative control (Figure 5.6A) to set the gate for Hoechst positive cells (infected RBC) (Figure 5.6B). To determine the number of nuclei per sample, the MFI of each sample was determined and expressed as a multiple of the MFI of one nucleus in one-hour old rings (Figure 5.7A). In the presence of IMP-1002, the WT parasite (PfNMT[G386]) population contained a reduced number of nuclei compared to the DMSO control (4-5 nuclei compared to 20 in the DMSO control), whilst PfNMT[G386E] parasites showed no difference between IMP-1002 treated parasites

at 140 nM and DMSO-treated control parasites (Figure 5.7B). The resistance of phenotypical inhibition through IMP-1002 by the G386E mutation further indicates on-target specificity of IMP-1002.

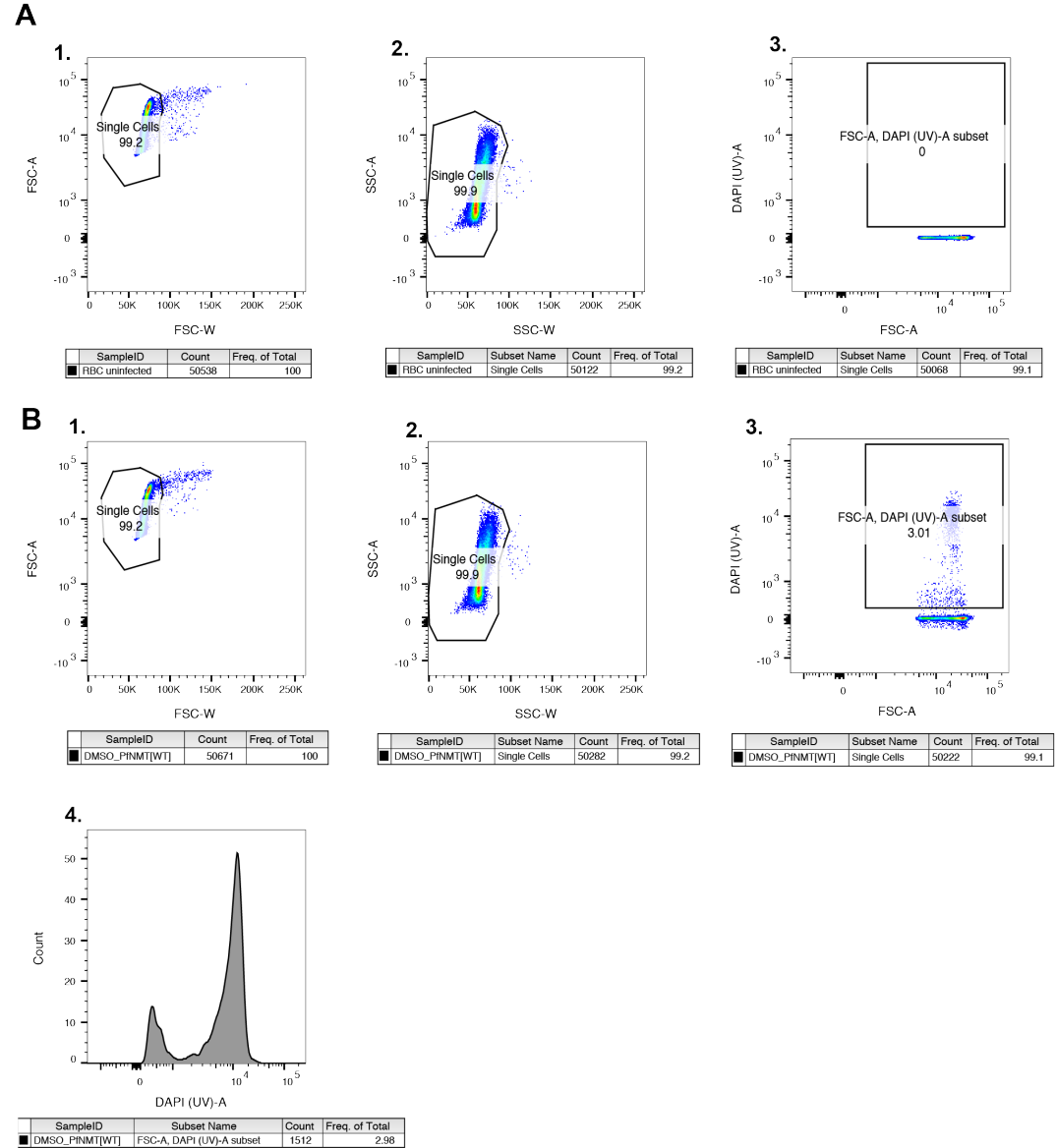


Figure 5.6 Gating strategy for the flow cytometry experiment.

A. The RBC only sample (uninfected RBC) was used as a control to set the boundaries between Hoechst positive and negative cells. B. The DMSO treated PfNMT WT sample is used as an example for a Hoechst positive sample. The gating strategy used for all samples was: FSC-A vs FSC-W (1) and SSC-A vs SSC-W (2) for doubled exclusion visualized in a dot plot, followed by 450-50/DAPI (UV)-A vs FSC-A to gate on Hoechst positive cells (3) which are subsequently shown (excluding the uninfected RBC population) using a histogram plot (4) with count vs 450-50/DAPI-UV/Hoechst (the final histogram is illustrated in figure 5.7A). A sample with synchronized rings was used to define the mean fluorescent intensity for parasites with one nucleus.

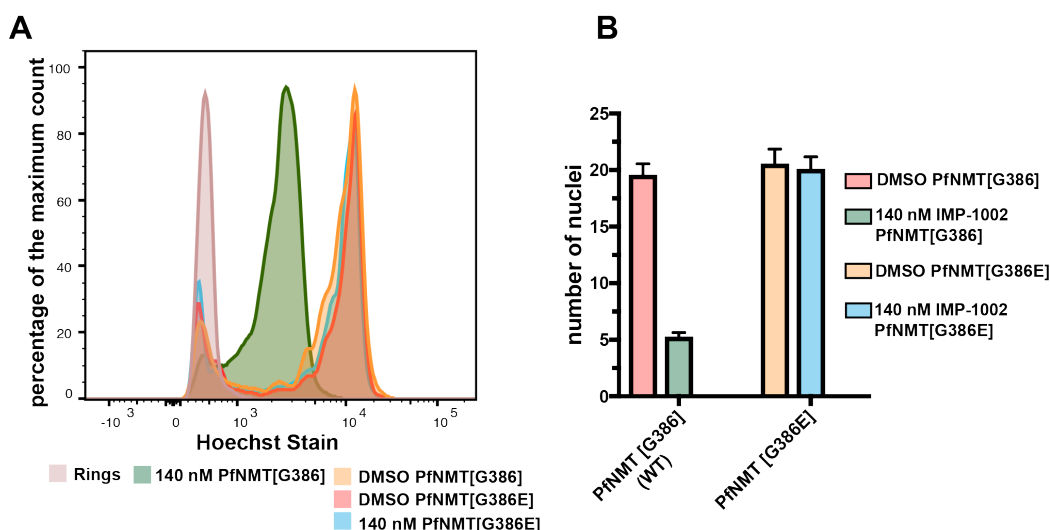


Figure 5.7 IMP-1002 shows on-target specificity through NMT inhibition phenotype reversion.

A) Percentage of maximum count of Hoechst-stained PfNMT[G386] and PfNMT[G386E] parasites used to determine the number of nuclei per sample. B) PfNMT[G386E] parasites show 0% inhibition at 140 nM. Graph shows flow cytometry data of Hoechst-stained parasites in A treated with 140 nM IMP-1002 from 1-45 h PI. The median fluorescent intensity (MFI) of the Hoechst-positive parasites was normalized to the MFI of parasites containing one nucleus (ring sample) in A to determine the average number of nuclei per sample. While WT parasites show a drop-in the number of nuclei and therefore an arrest in development at 140 nM NMTi, PfNMT[G386E] parasites show no arrest in development. All errors bars in this figure indicate standard deviation of the mean.

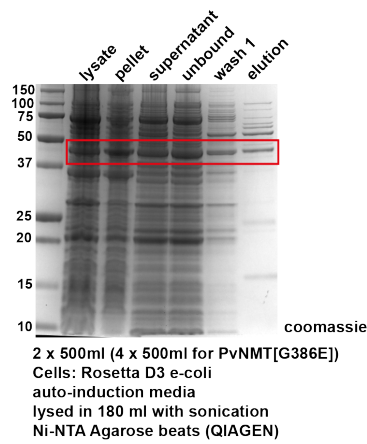
5.5 Enzymatic assay with PvNMT[G386E] shows refractory to binding and inhibition by IMP-1002

To clarify the biochemical basis for resistance to IMP-1002 *in vitro* expressed and purified *P. vivax* NMT (PvNMT) was used (see section 2.2.3.3). PvNMT possesses 80 % sequence identity (Table 1.1) and 93 % similarity to PfNMT and is an excellent platform for structural studies, whilst to date PfNMT has proven intractable for X-ray crystallography. Both PvNMT[G386] (Figure 5.8) and PvNMT[G386E] (purification not shown) were expressed in Rosetta-gami (DE3) *E. coli* and purified using a three step purification protocol of nickel affinity chromatography using the His-tag, anion exchange and size exclusion (see section 2.2.3.3) (Figure 5.8A-C). The overall yield was 832 µg/L for PvNMT[G386E] and 114µg/L for PvNMT[G386] which was measured by the absorbance of the protein at 280 nm through a full absorbance profile, resulting in a final concentration of 0.95mg/ml for PvNMT[G386E] and 0.76 mg/ml for PvNMT[G386] (Figure 5.8D). Both proteins still contained the His-tag

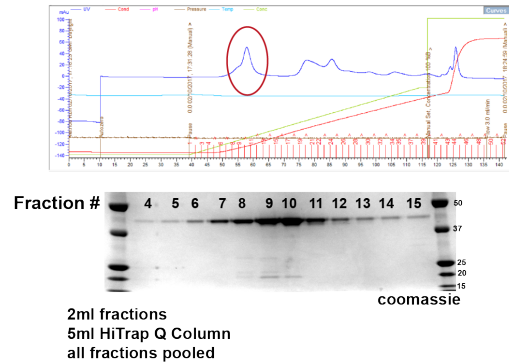
Chapter 5. A novel NMT inhibitor resistance breaking mechanism

(His-tag cleavage was only performed for the large-scale prep for crystallography), were concentrated, and showed similar purity (Figure 5.8E).

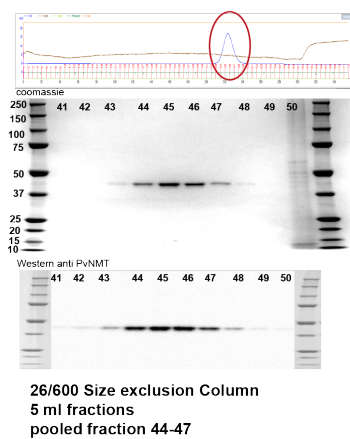
A Nickel purification PvNMT[G386]



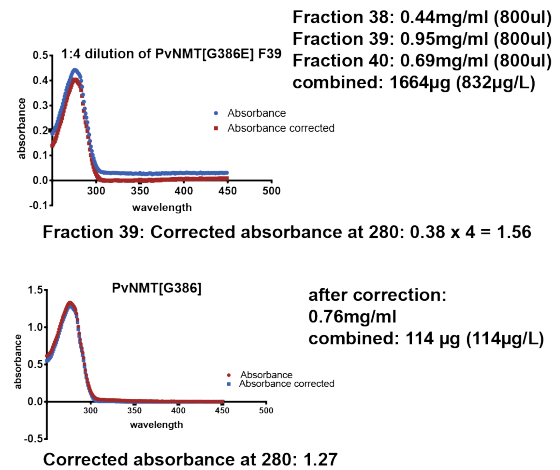
B Anion exchange chromatography PvNMT[G386]



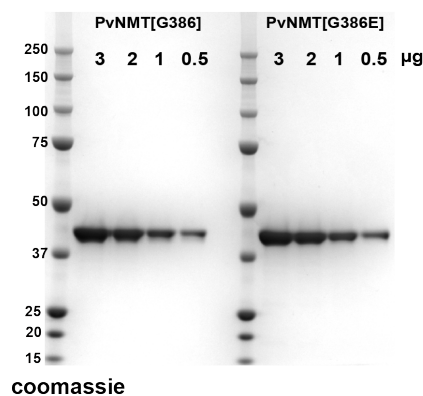
C Size exclusion PvNMT[G386]



D



E



F

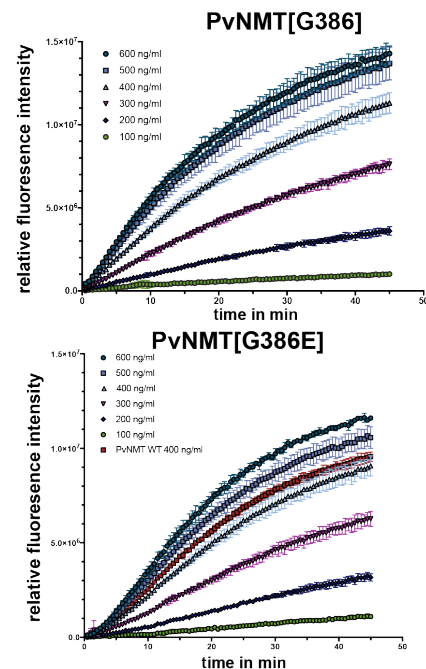


Figure 5.8 Expression and purification of PvNMT[G386] (WT) and PvNMT[G386E].

A. Nickel purification of PvNMT[G386] showing different fractions of the purification analysed by SDS-PAGE and Coomassie blue staining. Below are key details about the expression and nickel purification. B Anion exchange chromatogram of PvNMT[G386] elution. Elution peak of PvNMT[G386] is marked by a red circle and a Coomassie stained SDS-PAGE analysis of the pooled elution fractions is shown below the chromatogram. C. Size exclusion chromatogram of PvNMT[G386] elution. Elution peak of PvNMT[G386] is marked by a red circle. Eluted fractions were run on SDS-PAGE and analysed by coomassie blue staining and western blotting using an anti-PvNMT antibody. D. Full spectra absorbance of PvNMT[G386] and three selected fractions of PvNMT[G386E] (purification not shown) to determine corrected absorbance at 280 nm and calculate the protein concentration. The yield of each purification is stated at the right side of the graph. E. Different amounts of both proteins were run on an SDS-PAGE gel under reducing conditions to determine any contaminants. F. The activity of the enzyme was determined by the 7-diethylamino-3-(4-maleimido-phenyl)-4-methylcoumarin (CPM) assay with different amount of the enzyme.

Both enzymes were first tested for activity using the two substrates: Myr-CoA and the 15-residue NMT substrate peptide (HsSrc1) (see section 1.4.2) using the CPM assay. This assay uses a coumarin derivative thiol-reactive maleimide called 7-diethylamino-3-(4-maleimido-phenyl)-4-methylcoumarin (CPM) a fluorescent dye is used to monitor the formation of the coenzyme A thiol product (Goncalves, Brannigan, Thinon, et al. 2012) (see section 2.2.3.4). Activity was measured in a continuous assay over 45 min with different amounts of enzyme showing that both purified enzymes were active (Figure 5.8F). The kinetic profile between PvNMT[G386] and PvNMT[G386E] was compared for both substrates. For this, one substrate was used at saturating levels (30 μ M) while the other one was titrated to determine the kinetic profile in a continuous assay over 45 min using the same CPM assay (see section 2.2.3.4) (Figure 5.9). Both enzymes showed a similar kinetic profile with both the 15AA HsSrc peptide (Figure 5.9A) and MyrCoA (Figure 5.9B).

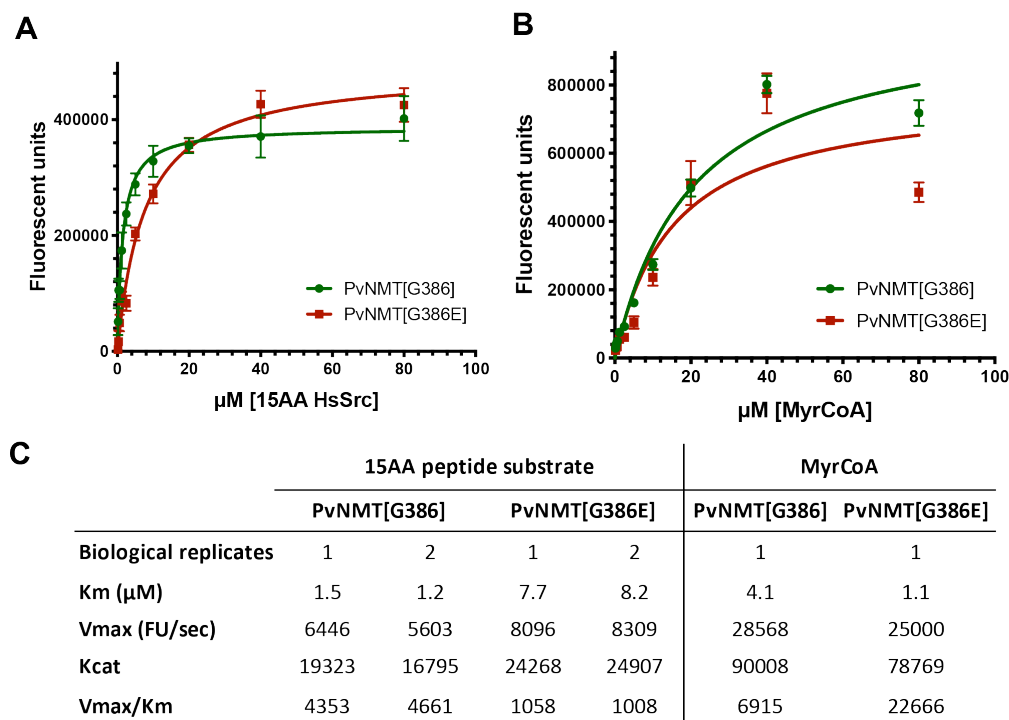


Figure 5.9 PvNMT[G386] and PvNMT[G386E] have similar kinetics for both substrates.

Michaelis-Menten plot for the K_m and V_{max} measurements for the 15-residue HsSrc peptide (A) and myristoyl-CoA substrate (B). During the continuous CPM assay one substrate was kept at saturating levels (30 μM) while the other was titrated to determine the K_m and V_{max} . (C) Table of K_m , V_{max} , K_{cat} and the catalytic efficiency (V_{max}/K_m) for the myrCoA and peptide kinetics. ($n = 2$ for peptide, $n = 1$ for MyrCoA).

Both PvNMT[G386] and PvNMT[G386E] were used to measure the binding affinity to NMTi IMP-1002 to determine a similar shift in binding affinity compared to the previously observed shift in parasite growth inhibition (Figure 5.3). The IMP-1002 IC_{50} was determined for both enzyme variants using the CPM assay (see section 2.2.3.4) which was quenched after 30 min. There was a significant 2.2-fold shift to higher concentration for the variant enzyme (Figure 5.10A) (Unpaired Welch T-test not assuming equal standard deviations, $p = 0.05$, $n = 3$). The CPM assay reaches its detection limit with the high potency of IMP-1002 resulting in a reduced shift in the variant enzyme. To complement the CPM assay a direct binding thermal shift assay with PvNMT[G386] and PvNMT[G386E] was performed (see section 2.2.3.5). This assay was performed by Adam Flinders at GSK. Consistent with the high affinity of IMP-1002 for the PvNMT[G386] this protein was stabilized by $> 8.8^\circ\text{C}$ with 5 μM of IMP-1002 even in the absence of Myr-CoA (Figure 5.10B). In contrast, the thermal stabilization with PvNMT[G386E] was only 1.8°C (Figure 5.10B). The same assay

was conducted in the presence of 4 μ M Myr-CoA, showing a similar trend (see Table 5.1 for raw data in section 5.7). However, the higher baseline melting temperature for the Myr-CoA-bound proteins compressed the difference stabilization window between the two proteins.

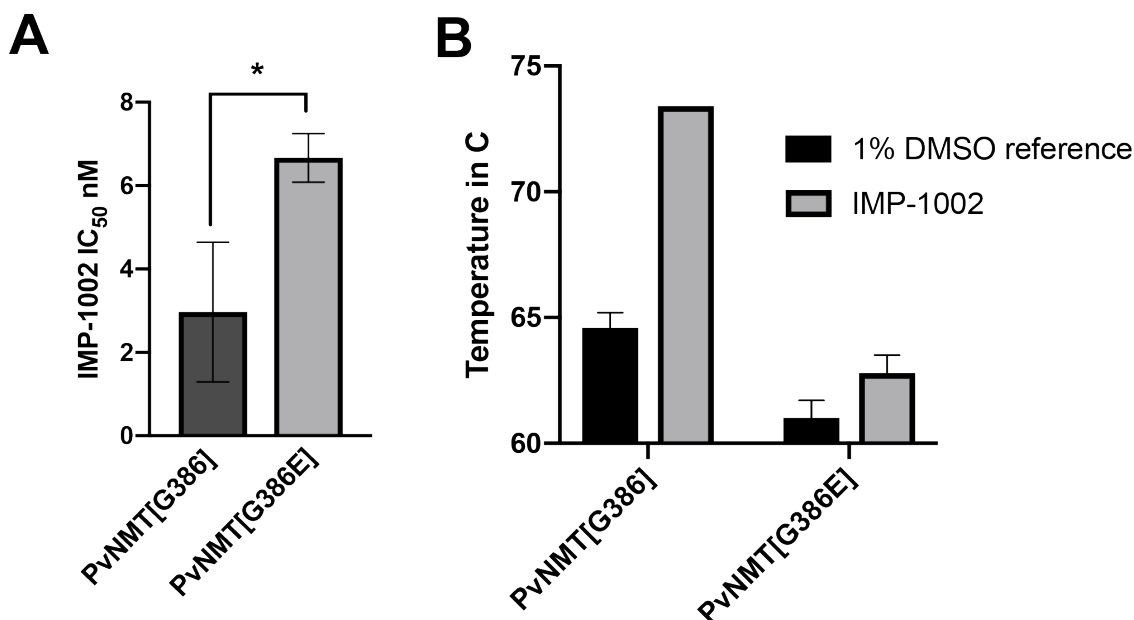


Figure 5.10 Consequences of G386E substitution for PvNMT function.

A. Bar graphs showing the effect of the G386E substitution on IC₅₀ in the biochemical assay. There is a significant shift in IC₅₀ induced though the G386E substitution. Unpaired Welch T-test not assuming equal standard deviations, $p = 0.05$, $n = 3$. For raw data please see table 5.3. B. Thermal shift in a protein stability assay with IMP-1002. The T_m was assayed without the presence of MyrCoA because a higher baseline T_m of PvNMT[G386] in the presence of MyrCoA led to compression of the difference window between PvNMT[G386] and PvNMT [G386E]. Since PvNMT[G386] bound to IMP-1002 was already at the top of the temperature range of the instrument without MyrCoA present, the data with MyrCoA were excluded. The data were acquired by Adam Flinders at GSK ($n = 2-3$). For raw data please see table 5.1. All errors bars in this figure indicate standard deviation of the mean.

5.6 X-ray crystallography identifies the structural basis for IMP-1002 resistance in PvNMT[G386E]

From previously published NMT co-crystal structures with different NMTi it is known that G386 is not in direct contact with any of the NMTi (see section 1.7.1) (Mousnier et al. 2018; Bell et al. 2012; Wright et al. 2014). It was therefore hypothesized that the resistance mechanism was based on an indirect conformation change in the

inhibitor binding site. The glutamate residue E386 is charged and larger than the glycine G386, which has no side chain, and is likely to interfere with the nearby Y211 residue. From comparisons of PvNMT and HsNMT, Y211 is known to adopt two different preferred rotamers, leading to different degrees of drug selectivity (Yu et al. 2012; Brannigan et al. 2014) (see section 1.7.1). Compounds which disfavour a certain preferred rotamer of Y211 for HsNMT can achieve a high degree of selectivity for parasite NMT. Binding of IMP-1002 shows good selectivity over the HsNMT through its tight π - π stacking interaction with Y211 but leaves little space for the larger glutamate residue at position 386. In order to test this hypothesis and understand the structural basis of resistance, Stephen Mayclin at the SSGCID in Seattle solved the co-crystal structure of PvNMT[G386] (PDB: 6MB1) and PvNMT [G386E] (PDB: 6MB0). There is minimal difference in the position of Y211 between the crystal structure of PvNMT[G386E] with bound Myr-CoA (but no inhibitor) (PDB: 6MAY) and that of PvNMT[G386] (PDB: 2ync) (Wright et al. 2014) (Figure 5.11A). The tyrosine indicates a certain degree of flexibility by adopting one of two observed rotamers (designated 'rotamer Y211a') in the PvNMT[G386E] protein crystal and both rotamers Y211a and Y211b in the PvNMT[G386]. Interestingly, the nearby H213 displays a larger degree of flexibility in the PvNMT[G386] structure, modelled as two possible rotamers (H213a, H213b), whereas the residue adopts a single conformation in the G386E variant (H213a). Upon IMP-1002 interaction with PvNMT[G386] (PDB: 6MB1), Y211 moves to a previously unobserved third rotamer conformation (Y211c) to form a π - π stacking interaction with the pendant phenyl ring of IMP-1002, whilst H213 remains in the unliganded position H213a (Figure 5.11B). Upon IMP-1002 binding to PvNMT[G386E], the glutamate side chain prevents formation of the more favourable π - π stacking interaction with the pendant phenyl ring, and forces H213 to move from rotamer position H213a to the more constrained H213b (Figure 5.11B). Both Y211 and H213 thus adopt alternative rotamers from those seen in PvNMT[G386] to create space for the inhibitor, leading to the loss of beneficial π - π stacking interactions consistent with the observed weaker affinity of IMP-1002 for the G386E variant (Figure 5.11B).

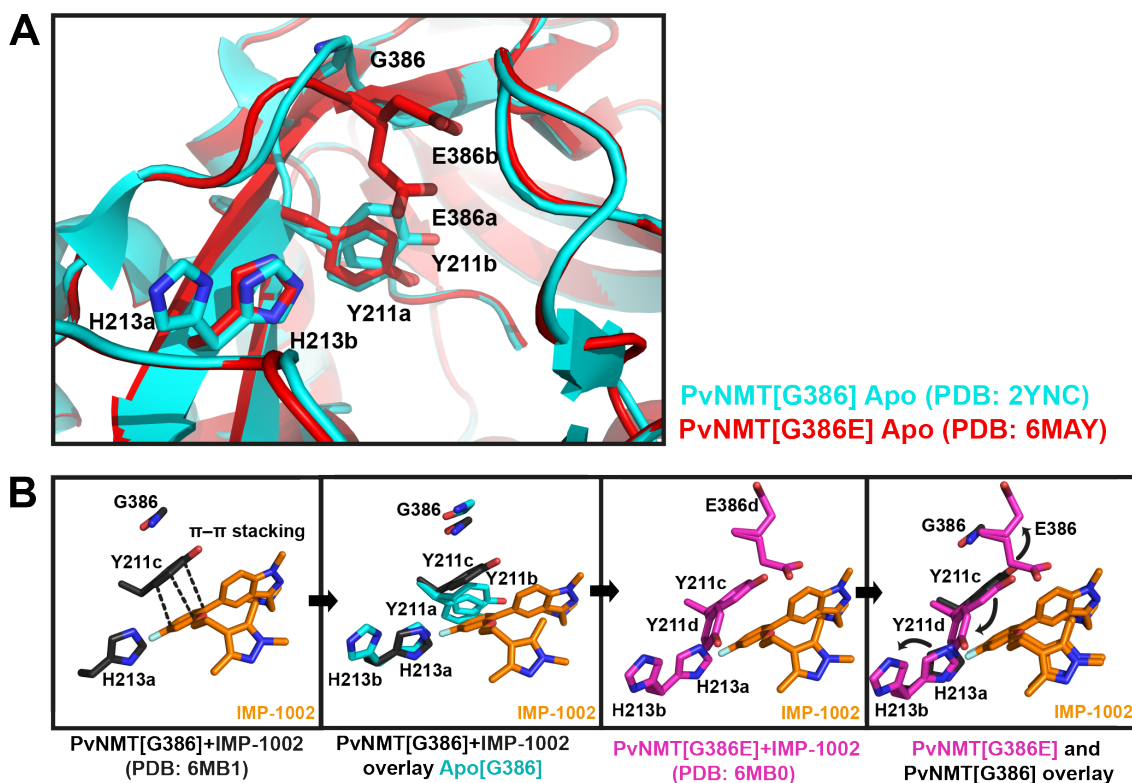


Figure 5.11 Crystallographic analysis of NMT inhibitor IMP-1002 interaction with PvNMT[G386] and PvNMT[G386E].

A. Crystal structure of PvNMT[G386] active site without inhibitor bound, overlaid with PvNMT[G386E] indicating flexibility of residue Y211 and H213 in peptide binding pocket. B. Movement of residues in the active site of PvNMT[G386E] compared to PvNMT[G386] upon interaction with IMP-1002. Both Y211 and H213 adopt alternative rotamers from those seen in PvNMT[G386] Apo to create space for the inhibitor, leading to the loss of beneficial π - π stacking interactions with IMP-1002 in the PvNMT[G386E] variant enzyme. Crystal structures were generated and solved by Stephen Mayclin at SSGCID. PDB IDs are given in the figure.

5.7 Predictive structure-guided identification of NMT inhibitor that overcomes G386E resistance both biochemically and in the parasite

To circumvent resistance the structural insights were used to explore different binding mode of NMTi which would deliver a resistance-breaking activity. DDD85646 is a recently described NMTi (Wright et al. 2014) (Figure 1.7) that binds to the peptide binding pocket with a different binding mode distinct to that of IMP-1002 (PDB ID: 2YND) and largely avoids direct interactions with Y211 (see section 1.7.1). This led to the hypothesis that DDD85646 would be unaffected by G386E. To test this hypothesis the same CPM assay (see section 2.2.3.4), thermal shift assay (see

section 2.2.3.5), and parasite growth assay (see section 2.2.1.10) were performed with DDD85646. To compare the data from both IMP-1002 and DDD85646 all values were expressed as a ratio of mutant variant over wild type variant. Both the EC_{50} (parasite growth assay) (Figure 5.12A) and IC_{50} (CPM assay) (Figure 5.12B) showed a ratio between 1 – 1.7 when comparing wild type and mutant enzyme (CPM assay - IC_{50} ratio = 1.4 ± 0.35 , growth assay - EC_{50} ratio = 1 ± 0.1) (Figure 5.12, for raw data see Table 5.2 and Table 5.3 in section 5.8). For the thermal-shift data performed by Adam Flinders, the shift in temperature between DMSO and each NMTi was first expressed as a ΔT_m by subtracting the T_m of the DMSO from the T_m of the bound NMT. The data were subsequently expressed as a $\Delta\Delta T_m$ subtracting the change of the mutant from the wild type. DDD85646 showed that the difference in stabilization ($\Delta\Delta T_m$) between PvNMT[G386] and PvNMT[G386E] was only $3^\circ C$, significantly less than the $>7.0^\circ C$ in favour of PvNMT[G386] observed for IMP-1002 (Figure 5.12C, for raw data see Table 5.1). The NMTi IMP-0320, was used as the GSK internal NMTi control in this thermal shift assay. Table 5.1 shows the binding of IMP-0320 to PvNMT[G386E] leads to a $>5.7^\circ C$ change in temperature over the DMSO control compared to $0.7^\circ C$ for PvNMT[G386]. This shows that the mutant enzyme is stabilized less by IMP-0320 compared to the wild type enzyme. The potential structural interaction with the G386E mutation of this compound is analysed in more detail in section 5.8.

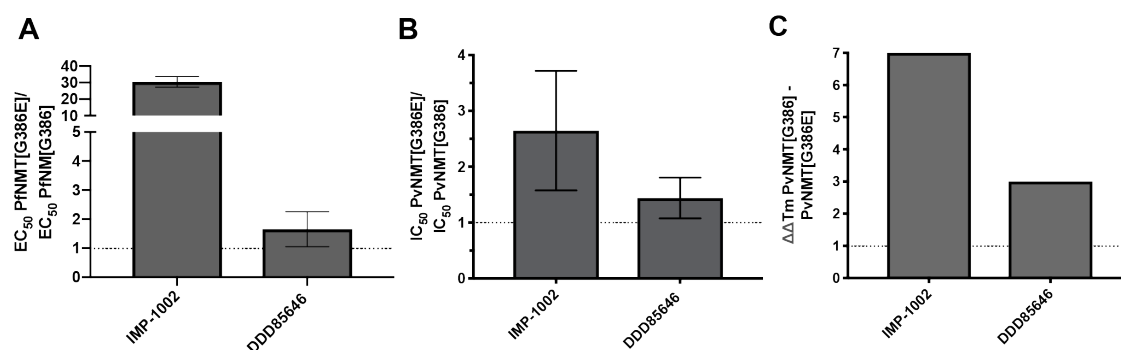


Figure 5.12 NMTi DDD85646 has a similar potency against P/NMT[G386E] and P/NMT[G386E].

Bar graphs showing the effect of the G386E substitution on (A) EC_{50} in the parasite growth assay, (B) IC_{50} in the biochemical assay and (C) thermal shift in a protein stability assay with IMP-1002 ($n = 2-3$). The substitution did not affect these values for the NMT inhibitor, DDD85646. All errors bars in this figure indicate standard deviation of the mean. For raw data please refer to table 5.2 (EC_{50}), table 5.3 (IC_{50}) and table 5.1 (thermal shift).

A

All T _m in °C	T _m	T _m	ΔT _m	ΔT _m	ΔΔT _m
Protein	PvNMT[G386] - PvNMT[G386E]				
- MyrCoA	PvNMT[G386]	PvNMT[G386E]	PvNMT[G386]	PvNMT[G386E]	PvNMT[G386E]
Compound ↓					
1% DMSO reference	64.6 ± 0.6	61.0 ± 0.7	0	0	0
IMP-0320	>70.4 [#]	61.7 ± 0.8	>5.7	0.7	>5.0
DDD85646	69.0 ± 0.1	62.4 ± 0.7	4.4	1.3	3
IMP-1002	>73.4 [#]	62.8 ± 0.7	>8.8	1.8	>7.0

Protein was not fully denatured at the end of the thermal ramp, therefore these values represent the lower estimates of T_m

B

All T _m in °C	T _m	T _m	ΔT _m	ΔT _m	ΔΔT _m
Protein	PvNMT[G386] - PvNMT[G386E]				
+ 4 μM MyrCoA	PvNMT[G386]	PvNMT[G386E]	PvNMT[G386]	PvNMT[G386E]	PvNMT[G386E]
Compound ↓					
1% DMSO reference	67.8 ± 0.6	61.6 ± 0.5	0	0	0
IMP-0320	>73 [#]	63.2 ± 2.2	>5.2	1.6	>3.6
DDD85646	71.0 ± 0.1	63.0 ± 0.3	3.2	1.4	1.8
IMP-1002	>75 [#]	66.6 ± 0.6	>7.2	5	>2.2

Protein was not fully denatured at the end of the thermal ramp, therefore these values represent the lower estimates of T_m.

Table 5.1 Thermal shift assay raw data.

The degree of stabilisation of PvNMT[G386] and PvNMT[G386E] was determined using at least two experimental replicates with two technical replicates (n>5) with three different NMTi compounds in the absence (A) and (B) presence of (MyrCoA). All T_m values are given in °C and ΔT_m is determined by subtracting the T_m for the DMSO control to determine the compound induced stabilisation each protein. There is significantly greater stabilisation of the PvNMT[G386] for compounds such as IMP-0320 (GSK internal control) and IMP-1002 over PvNMT[G386E], as evident from the ΔΔT_m values. For the PvNMT[G386], where the T_m of the compound bound protein exceeds the top of the temperature range for the instrument, a greater than (>) value for the T_m is given. These lower estimates of T_m may lead to compression of the difference window between PvNMT[G386] and PvNMT [G386E] for IMP-0320 and IMP-1002. This is most noticeable with the higher baseline T_m of PvNMT[G386] in the presence of MyrCoA. Data acquired by Adam Flinders at GSK.

In collaboration with SSGCID, the crystal structure of PvNMT[G386E] bound to DDD85646 was solved (PDB: 6MAZ) and compared to the previously published structure of PvNMT[G386] bound to DDD85646 (PDB: 2YND) (Wright et al. 2014). This overlay showed that there was only a minimal movement of either Y211 or H213 in the inhibitor-bound form of PvNMT[G386E] compared to PvNMT[G386], confirming the molecular basis for similar affinity (Figure 5.13A). Structural analysis with both molecules demonstrated that the position off all three amino acids involved

in the resistance mechanism (E386, Y211 and H213) were influenced by the binding mode IMP-1002 but not DDD85646 (Figure 5.13A, Figure 5.11). By including the PvNMT[G386] structure in the comparison, Y211 was observed to adopt at least four different conformations. A preferred conformation (rotamer Y211a) was adopted in both the unliganded PvNMT[G386] and [G386E] enzymes, and upon binding of DDD85646 to PvNMT[G386] (Figure 5.13B). Y211 moved only slightly, to rotamer Y211b, when DDD85646 was bound to PvNMT[G386E]. For PvNMT[G386] with bound IMP-1002, Y211 adopts rotamer Y211c, allowing the π - π stacking interaction with IMP-1002 that likely results in the increased potency and selectivity (over HsNMT) of this compound compared to DDD85646 (Figure 5.13B). However, for PvNMT[G386E] with bound IMP-1002, Y211 moves to rotamer position Y211d to avoid a steric clash with E386: this restricts the ability of Y211 to adopt its preferred conformation, preventing the π - π stacking interaction with IMP-1002 and leading to the shift of H213 from H213a to H213b (Figure 5.13B). Figure 5.13B also shows the considerable flexibility of E386 in the different structures. Depending on the inhibitor bound (IMP-1002, DDD85646 or in the apo form) E386 can adopt up to four distinct occupancies showing its flexible nature (Figure 5.13B).

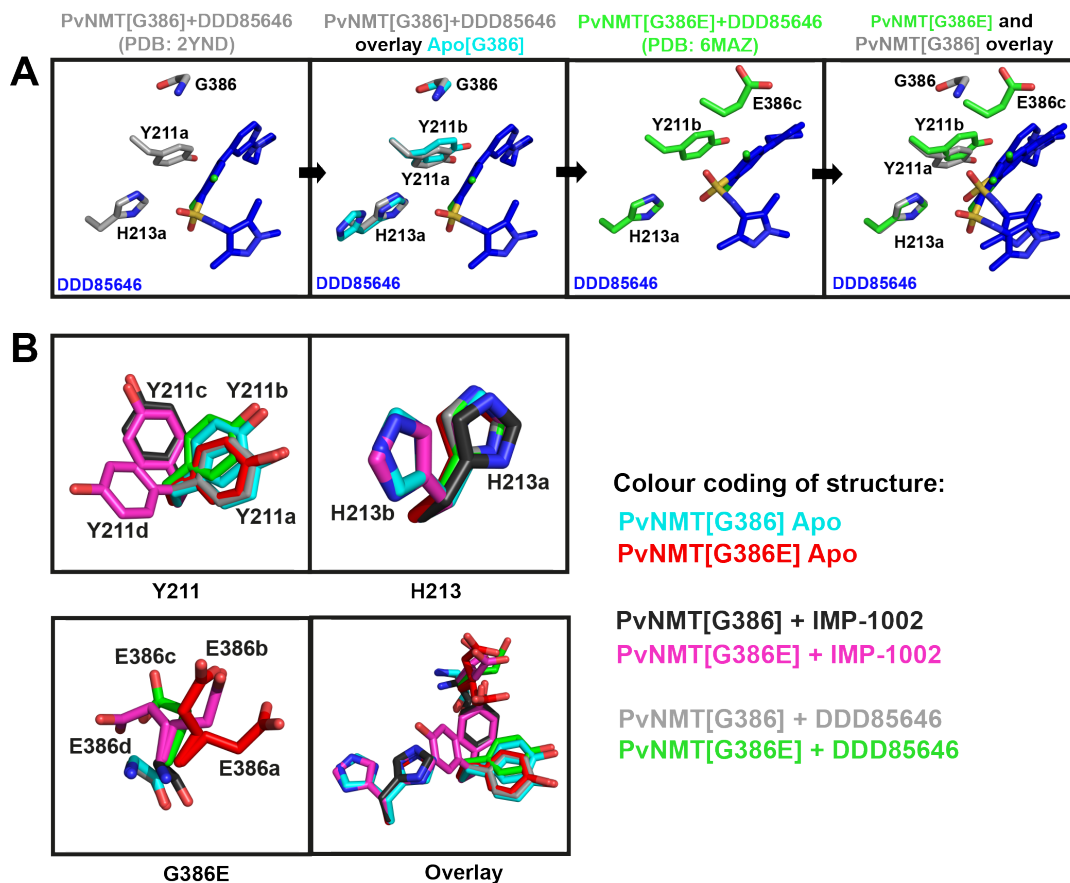


Figure 5.13 Crystallographic analysis of NMT inhibitor DDD85646 interaction with PvNMT[G386] and PvNMT[G386E].

A. Movement of residues in the active site of PvNMT[G386E] compared to PvNMT[G386] upon DDD85646 binding B. Y211 movement in the active site in the six different crystal structures. Y211 can adopt four distinct rotamers depending on the G386E mutation and type of inhibitor bound. This is followed by H213 that can adopt two different rotamer occupancies to accommodate the extra space taken up by Y211 when G386 is mutated to E386. Crystal structures were generated and solved by Stephen Mayclin at SSGCID. PDB IDs are given in the figure.

Overall the minimal disruption of Y211 and H213 by DDD85646 binding results in the conserved affinity and binding mode for both WT and G386E variants (Figure 5.13), and these data together with minimally perturbed IC_{50} , EC_{50} and thermal stability (Figure 5.12) lead us to conclude that DDD85646 can break resistance in NMT[G386E] parasites.

5.8 The Impact of G386E on different NMT inhibitors

The binding mode of other NMTi from different series were further analysed using published crystal structures of PvNMT[G386] (wild type) with bound NMTi, IMP-0320 (PDB ID: 2YNE) (Wright et al. 2014) and IMP-0856 (PDB ID: 4UFX) (Yu et al. 2012) (for structures of compounds see Figure 1.7) overlaid with the PvNMT[G386E] structure (Figure 5.14). The location of the compound binding to the peptide binding pocket and its proximity to G386E and Y211 was assessed and to hypothesize if the binding of these inhibitors would be affected by the G386E substitution. The movement of Y211 from position Y211c to Y211d accompanied by the prevention of favourable interactions with both inhibitors, led to the hypothesis that their binding would also be affected by the G386E substitution, unlike DDD85646. To test this, the same *in vitro* EC_{50} shift assay was performed in combination with the IC_{50} determination with the CPM assay using IMP-0964 (a close derivative of IMP-0856) and IMP-0320. Shifts in EC_{50} (Figure 5.15A, Table 5.2) correlated very well with shifts in IC_{50} (Figure 5.15B, Table 5.3). These results confirmed that binding of both IMP-0320 and IMP-0964 were affected by the G386E substitution. In the same experiments, the potency of DDD85646 in killing parasites in the cell-based assay (Figure 5.15A) and binding in the enzyme assay (Figure 5.15B) was unaffected by the G386E change.

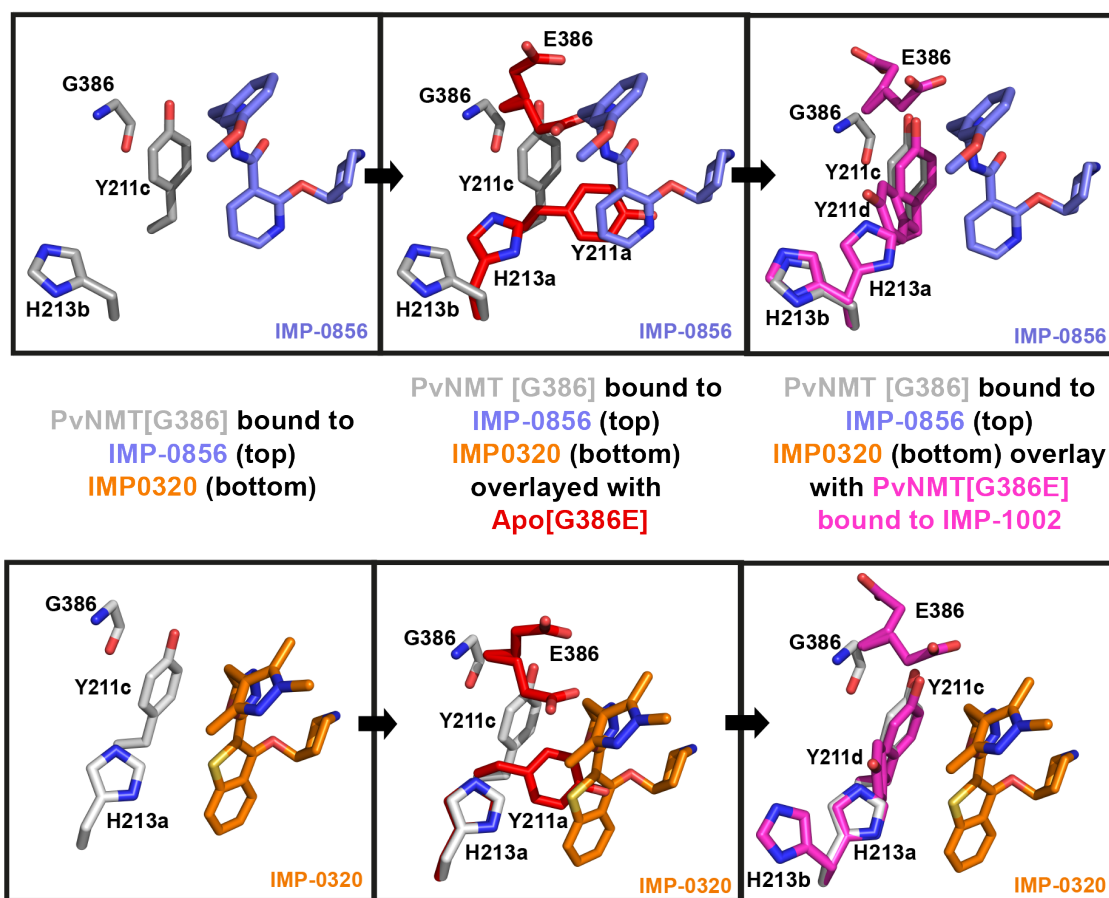


Figure 5.14 Crystallographic analysis of NMT inhibitor interaction with PvNMT[G386] and PvNMT[G386E].

Movement of residues as a result of the G386E mutation upon binding of two different NMT inhibitors: IMP-0856 at the top (PDB ID: 4UFX) and IMP-0320 (PDB ID: 2YNE) at the bottom. First image shows the PvNMT[G386] with respective inhibitor bound. Upon overlay with Apo[G386E] it becomes apparent that Y211 can no longer position in the Y211c rotamer because of the G386E substitution, and the Y211a rotamer cannot be adopted with IMP-0320 or IMP-0856 bound. Therefore, in the last image it is predicted that a PvNMT[G386E] bound to the respective NMTi would have similar characteristics as PvNMT[G386E] bound to IMP-1002 (PDB: 6MB0) with movement of Y211 from Y211c to Y211d.

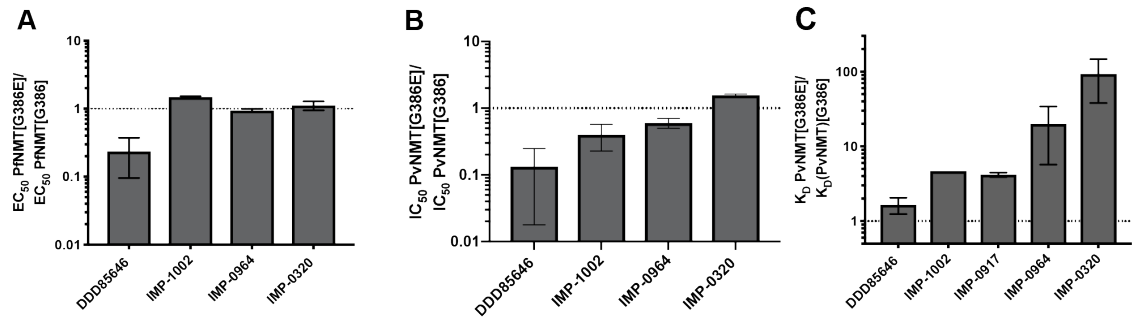


Figure 5.15 The NMT[G386E] variant is affected differently by a range of NMT Inhibitors.

A. Mean increase of EC_{50} between PfNMT[G386E] and PfNMT[G386] parasites measured through SYBR Green growth assay (visualized on a log scale) ($n = 2$). A mean increase of 10 is indicated by a dotted line. B. Enzymatic CPM assay measuring the mean increase in IC_{50} with different NMTi with purified PvNMT[G386] and PvNMT[G386E] (visualized on a log scale) ($n = 3$). A mean increase of 10 is indicated by a dotted line. C. Mean increase in binding affinity of NMTi for PvNMT[G386] and PvNMT[G386E] (visualized on a log scale) ($n = 1-3$). The K_D of each compound with PvNMT[G386E] was divided by the K_D with PvNMT[G386] to calculate the fold difference. K_D is the equilibrium dissociation constant of NMTi calculated from fitted response-concentration plots measured by SPR analyses. Only one K_D could be accurately determined for IMP-1002 and so error bars are not shown. A mean increase of 10 is indicated by a dotted line. All errors bars in this figure indicate standard deviation of the mean.

Growth assay (EC_{50} determination)

	PfNMT[G386]			PfNMT[G386E] Clone 1			PfNMT[G386E] Clone 2			Ratio PfNMT[G386] / PfNMT[G386E]	
	EC_{50} (nM)	95% CI (nM)	Hill Slope	EC_{50} (nM)	95% CI (nM)	Hill Slope	EC_{50} (nM)	95% CI (nM)	Hill Slope	Clone 1	Clone 2
IMP-1002	9.6	8.2 - 11	-2.3	230	190 - 280	-1.7	201	170 - 250	-1.5	24	21
DDD85646	69	51 - 93	-0.65	91	75 - 110	-1.4	103	86 - 120	-1.5	1.3	1.5
IMP-0964	93	83 - 103	-2.9	1080	913 - 1300	-1.3	947	78 - 83	-0.89	12	10
IMP-0320	80	68 - 93	-2.6	1500	1200 - 1800	-1.4	1400	1100 - 1900	-0.91	19	18

Table 5.2 Raw data for SYBR Green growth assay.

SYBR Green growth assay data to determine the EC_{50} of PfNMT[G386] and PfNMT[G386E] parasites with two biological replicates in the form of two clones from independent transfections with different guides. All numbers are given to two significant figures. In the last column the ratio is calculated of the EC_{50} of PfNMT[G386E]/PfNMT[G386].

CPM enzyme assay (IC ₅₀ determination)								
	PvNMT[G386]				PvNMT[G398E]			Ratio PvNMT[G386E]/ PvNMT[G386]
	Replicate	IC ₅₀ (nM)	95% CI (nM)	Hill Slope	IC ₅₀ (nM)	95% CI (nM)	Hill Slope	
IMP-1002	1	1.9	1.6 - 2.1	-2.1	6.0	5.4 - 6.5	-1.8	3.1
	2	2.1	2.0 - 2.3	-1.7	7.1	2.3 - 41	-0.82	3.4
	3	4.9	4.4 - 5.4	-2.0	6.9	6.2 - 7.7	-1.5	1.4
DDD85646	1	53	38 - 65	-0.9	55	41 - 62	-0.9	1.0
	2	47	37 - 66	-1.1	77	61 - 110	-1.5	1.6
	3	48	40 - 62	-1.1	80	52 - 204	-0.9	1.7
IMP-0964	1	3.7	3.3 - 4.2	-1.2	21	18 - 24	-1.2	5.5
	2	4.7	2.3 - 7.2	-3.6	17	12 - 24	-1.3	3.6
	3	12	12 - 13	-1.9	35	33 - 38	-1.5	2.9
IMP-0320	1	10	9.2 - 11	-1.2	380	180 - 4900	-0.9	38
	2	9.1	8.5 - 10	-1.3	340	140 - 1800	-0.9	37
	3	10	9.4 - 11	-1.3	300	150 - 6800	-1.0	29

Table 5.3 Raw data for CPM enzyme assay.

CPM enzyme assay data to determine the IC₅₀ of PvNMT[G386] and PvNMT[G386E] with three biological replicates of four different NMTi compounds. All numbers are given to two significant figures. The Hill Slope of the IC₅₀ gives an indication how steep the curve is. For very high hill slopes < -1, the IC₅₀ value cannot be accurately determined and is presumably lower than indicated. In the last column the ratio is calculated of the IC₅₀ of PvNMT[G386E]/PvNMT[G386].

To complement the CPM assay and potentially increase the accuracy of measuring IC₅₀ values below 10 nM in the case of highly potent compounds, the surface plasmon resonance (SPR) assay was used to determine the relative K_D values of different NMTi with the two forms of the enzyme (see section 2.2.3.6). The NMTi IMP-0917, a close derivative of IMP-1002 that has recently been published (Mousnier et al. 2018) (Figure 1.7), was also included in the SPR assays, and was expected to behave like IMP-1002 due to its similar structural properties. The SPR experiment were carried out at GSK in Stevenage with the help of Chun-wa Chung and David Charter. The assay was configured so that the same concentration titrations were flowed over the surfaces immobilized with both proteins and a blank flow cell control. To focus on the selectivity between proteins and remove inter experiment variability, the ratio of K_Ds between the two proteins was calculated in each experiment (n = 1-4) as this allows better differentiation between differences in affinity (Figure 5.15C, Figure 5.16, Table 5.4). The SPR data were collected at very low signal to background ratio to avoid mass transport limitations whereas at high levels of protein bound the rate at which the compound binds to the protein would exceed the rate at which the compound can be delivered to the surface, making mass

transport the rate limiting step. Secondly there is a higher issue of compound rebinding to unoccupied protein before defusing off when the flow cell is washed. Originally the goal was to extract kinetic measurement from the sensorgrams but this was not possible especially for IMP-1002 with PvNMT[G386] as kinetics were too fast to be correctly determined (Figure 5.16). Therefore, only the equilibrium steady state values were fitted as the equilibrium model is independent of the binding mechanism.

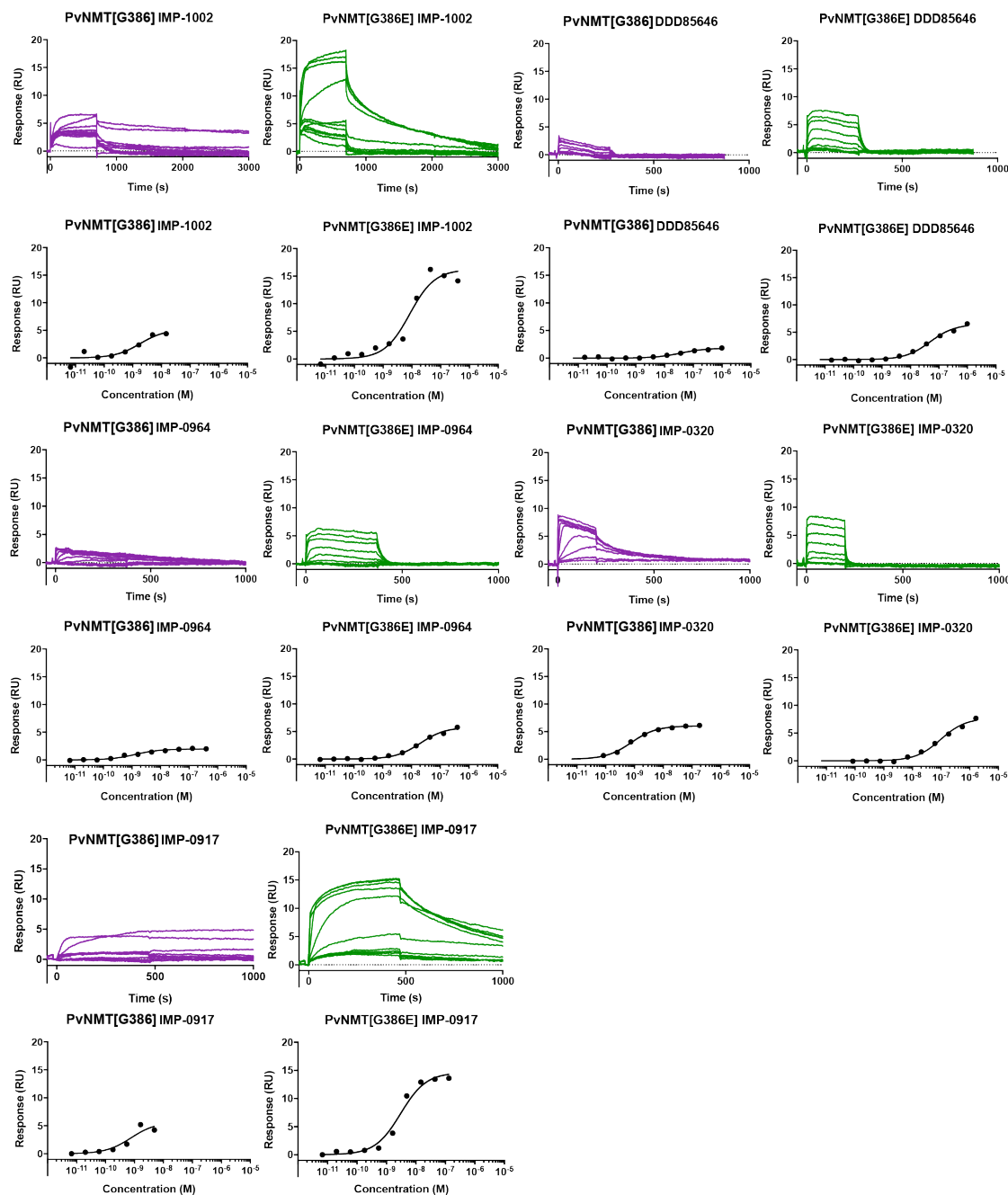


Figure 5.16 Surface plasmon resonance analysis of the interaction of NMTi with PvNMT[G386] and PvNMT[G386E].

The relationship between each point in the concentration series and the report points from sensorgrams measured for each of the NMTi compounds was fitted to a 1:1 steady state affinity model to calculate the equilibrium dissociation constant (K_D).

A

Compound	PvNMT[G386] K_D (expt1)	PvNMT[G386] (expt2)	PvNMT[G386] (expt3)	PvNMT[G386] (expt4)
IMP-0320	7.79E-10	2.41E-09	7.47E-09	6.87E-10
DDD85646	3.61E-08	2.96E-08	1.51E-08	2.18E-08
IMP-0964	1.29E-09	6.47E-10	8.17E-09	3.66E-10
IMP-1002	1.80E-09			
IMP-0917	6.48E-10	7.52E-10		

B

Compound	PvNMT[G386E] K_D (expt1)	PvNMT[G386E] (expt2)	PvNMT[G386E] (expt3)	PvNMT[G386E] (expt4)
IMP-0320	9.72E-08	1.88E-07	1.68E-07	9.95E-08
DDD85646	4.90E-08	4.47E-08	2.23E-08	4.91E-08
IMP-0964	2.07E-08	1.64E-08	1.52E-08	1.22E-08
IMP-1002	8.37E-09	4.83E-09	3.63E-09	2.98E-09
IMP-0917	2.58E-09	3.29E-09		

C

Compound	PvNMT[G386E]/ PvNMT[G386] K_D (expt1)	PvNMT[G386E]/ PvNMT[G386] (expt2)	PvNMT[G386E]/ PvNMT[G386] (expt3)	PvNMT[G386E]/ PvNMT[G386] (expt4)
IMP-0320	77.8	22.5	124.8	144.9
DDD85646	1.5	1.5	2.3	1.4
IMP-0964	25.3	41.5	16.1	11.7
IMP-1002	4.7			
IMP-0917	4	4.4		

D

Compound	K_D (PvNMT[G386E]) / K_D PvNMT[G386]
IMP-0320	92.5 ± 54.5
DDD85646	1.6 ± 0.4
IMP-0964	19.9 ± 14.2
IMP-1002	4.7 (n = 1)
IMP-0917	4.2 ± 0.3

Table 5.4 Data table for SPR experiment with PvNMT[G386] and PvNMT[G386E].

Fold difference of equilibrium dissociation constants (K_D) determined from SPR analyses for the interaction between each NMTi compound with PvNMT[G386] or PvNMT[G386E]. A. Raw data of K_D values for PvNMT[G386] (n = 1-4) and B. PvNMT[G386E] (n = 2-4). C. The SPR experiment was configured so that the same concentration titrations were flowed over surfaces immobilized with the three proteins. To focus on the selectivity between proteins and remove inter-experiment variability, the ratio of K_D s between the three proteins was calculated in each experiment (n = 1-4) as this allows better differentiation between differences in affinity. D The K_D of each compound with either PvNMT[G386E] was divided by the K_D with PvNMT[G386] to calculate fold difference. The standard deviation of the mean fold difference is stated.

From the data it was possible to determine that the affinities of IMP-1002 and IMP-0917 decreased by approximately 5-fold because of the G386E substitution, whereas binding of DDD85646 was relatively insensitive. IMP-0320 and IMP-0964 showed the most marked decrease in affinity, almost 100 and 20-fold respectively between the PvNMT[G386E] and PvNMT[WT] protein (Figure 5.16, Table 5.4, Figure 5.15C). The kinetic differences between the compounds with the PvNMT[WT] and PvNMT[G386E] enzymes reflected this generally weaker binding, with faster off rates apparent from the sensorgram traces. IMP-1002 and IMP-0917 exhibited interesting kinetics: both showed a slow dissociation rate with PvNMT[G386E] (Figure 5.16) suggesting that several hours may be required for complete dissociation. Although the effect was largest for IMP-1002, the long dissociation rates of both compounds probably lead to an underestimate of their affinities due to reduced binding capacity in sequential injections during a standard equilibrium K_D SPR analysis. In combination with the shift in EC_{50} , IC_{50} , and thermal stability, the SPR data clearly show that DDD85646 can overcome resistance mediated by G386E.

5.9 Discussion

This chapter describes the identification and validation of the consequence of a single amino acid substitution (G386E) in the substrate-binding pocket of *Plasmodium* NMT, identified by drug selection of a non-synonymous point mutation in the *nmt* open reading frame. This mutation is the first identified to date for this drug target and the importance was confirmed by CRISPR-Cas9 mediated substitution of this single nucleotide in the WT parasite, recapitulating resistance against three chemically distinct NMT inhibitor series. Data from the PfNMT[G386E] resistant parasite line were used to guide biochemical and structural analysis of this variant in recombinant PvNMT, providing both enzymatic inhibition data and the crystal structures of the WT and G386E variant with bound Myr-CoA and the NMTi used for mutation selection. Structural, IC_{50} and K_D data for PvNMT[G386E] are consistent and correlated with the EC_{50} shift data obtained in G386E parasites, although the active site of PvNMT differs from PfNMT in 2 out of 23 residues (F212Y and F334Y, PfNMT to PvNMT) (Yu et al. 2012). These structural data provide insights into changes in the active site induced by the G386E substitution, including the modes of

binding for different inhibitors and the flexibility required within the peptide-binding pocket to accommodate them. Using these data, a structure-guided approach was used to identify compounds that can overcome resistance mediated by the G386E substitution, exploiting the differential binding mode of DDD85646. This predictive structural model can guide further discovery of drugs active against NMT, and current work is focused on optimizing a new lead inhibitor series unaffected by G386E. The identification of NMTi that circumvent the G386E mutation suggests opportunities to combine different inhibitors to overcome selective resistance, and thus thwart the emergence of novel resistant variants.

Interestingly, the G386E variant parasite shows no apparent reduction of fitness in growth at the asexual blood stage, although it is currently unknown whether this observation would apply throughout the complete parasite life cycle. Even though G386E reduces the space in the peptide binding pocket, the flexibility of Y211, E386 and H213 (Figure 5.11) provides a plausible explanation for why substrate myristoylation is unaffected, leading to no reduction in fitness or change in global myristoylation (Figure 5.4). Future studies on the potential of various substrates with different sequences downstream of the N-terminal glycine to differentially stabilise intermediate states during the catalytic cycle of NMT, in combination with studies on the flexibility of turn-over of different substrates in the WT and G386E variant NMTs, could give further insights into substrate specificity.

Together, these data provide direct genetic evidence for the specific on-target activity of each of the NMTi classes described in this chapter, which in combination with the previously reported chemical biology studies (see Chapter 3) further supports the validity of this target in malaria. The relatively fast selection of resistance to NMTi may suggest the potential for rapid development of resistance in the clinic. However, the SNP responsible for G386E is not present in any of the field isolates catalogued in PlasmoDB. In the 218 available isolates, there are seven non-synonymous mutations in the coding region resulting in six amino acid changes. The first four amino acids of the N-terminus of NMT have all been mapped with a possible SNP, but this is most likely because of a mixed infection indicated by a mixture of SNP and wild type sequence in the reads and poor sequence coverage resulting from the AT-rich 5' UTR. Two SNPs with high and robust sequence coverage are in codons 110

and 227 (Glu₁₁₀Asp and Leu₂₇₇Phe), but both amino acid changes are distal to the substrate-binding site and unlikely to interfere with NMTi binding based on their location. The repeated selection of the G386E mutant in the Dd2 cloned line over this very short period of drug pressure may imply the rapid acquisition of this mutation *in vivo*. It would therefore be expected to see this mutation arising in the field when drug pressure is applied, considering that there was no fitness cost associated with G386E *in vitro*. Structural biology has been applied extensively to drug resistance studies, including to target based drug discovery against malaria to improve drug affinity for drug targets like for cytochrome bc1, NADH dehydrogenase (NDH2) (Biagini et al. 2012), dihydroorotate dehydrogenase (DHODH) (Coteron et al. 2011) and dihydrofolate reductase (DHFR) (Yuthavong et al. 2012) (see section 1.6.4). Structure guided drug discovery has also been applied in other fields including influenza (Y. Wu et al. 2016), and hepatitis C (Lagacé et al. 2011) highlighting the general importance of structure-guided design for the discovery of resistance-breaking inhibitors.

In the future it would be of interest to attempt selection of parasites resistant to DDD85646, since the on-target activity of DDD85646 (compound 1a in (Wright et al. 2014)), rules out an off-target killing of PfNMT[G386E] parasites. DDD85646 binding is insensitive to the resistance mechanism generated in response to IMP-1002 treatment, but at a cost of lower selectivity over HsNMT (Table 5.5) since Y211 needs to move from the 'in' Y211c rotamer occupancy which provides improved selectivity over HsNMT (Yu et al. 2012), to the 'out' Y211d rotamer, leaving room for G386E.

	HsNMT1	HsNMT2	PvNMT	HsNMT1/PvNMT	HsNMT2/PvNMT
IMP-1002	< 0.0072 $\times/\div$ 3.7821 (n=4)	0.0119 $\times/\div$ 2.3488 (n=3)	< 0.0031 $\times/\div$ 2.5220 (n=7)	2.32	3.84
DDD85646	0.0127 $\times/\div$ 1.4054 (n=3)	0.0200 (n=1)	0.0407 $\times/\div$ 1.6927 (n=5)	0.3	0.5

Table 5.5 IC₅₀ of HsNMT1 HsNMT2 and PvNMT with IMP-1002 and DDD85646 determined through CPM assay.

Values are concentrations in μ M. Values are the geometric mean with associated standard error showing the variation from the biological replicates. The last two columns highlight the fold difference in IC₅₀ of PvNMT compared to HsNMT1 and HsNMT2. While there is a 2-3 fold shift in selectivity towards PvNMT with IMP-1002 this selectivity is diminished for DDD85646. Assays on HsNMT1/2 were conducted by Markus Ritzefeld.

Future studies will focus on identification of an inhibitor series that can overcome the G386E-mediated resistance without compromising selectivity, based on either IMP-1002 analogues or more likely a new chemical series with a distinct binding mode. For example, the series that includes IMP-1002 has recently been adapted to increase HsNMT selectivity with a relatively simple adaptation (Mousnier et al. 2018). This shows the flexibility of the series has the scope to find analogues of IMP-1002 that can also overcome G386E-mediated resistance. In summary, this structure-guided analysis provides mechanistic rationalization and enables discovery of an NMTi that can overcome the first identified NMT resistance mechanism resulting from the G386E substitution. This approach defines a new strategy to overcome acquisition of NMTi resistance, and will facilitate further drug development efforts.

Chapter 6. Conclusion and Future Perspectives

NMT is a constitutively expressed enzyme which carries out a highly specific modification on many different substrates involved in diverse pathways with essential functions for *P. falciparum* biology. The enzyme is essential for the malaria parasite shown through genetic validation in *P. berghei* (Pino et al., 2012) and through the use of selective chemical compounds in *P. falciparum* (see Chapter 3). It has been genetically and chemically validated as a drug target in many infectious diseases including *T. brucei* (Frearson et al. 2010), *T. cruzi* (Herrera et al. 2016), *L. major* (Tate et al. 2013; Wright et al. 2015), and *Plasmodium* (Wright et al. 2014) and studies have started to validate it as a drug target in *Toxoplasma gondii* (Alonso et al. 2019). The current inhibitors designed against PfNMT and PvNMT have picomolar activity, act on target, have a fast killing rate *in vitro* within the first cycle of the parasite and have no known cross-resistance to other current drugs. In addition to the validation of NMT as a promising drug target against malaria, it is important to determine the pharmacokinetic profile of the NMT inhibitors and their potential usage *in vivo* as well as later in the clinic. Although not part of this thesis, the work that has been done in the past with Medicine for Malaria Venture (MMV) and currently in collaboration with GSK is important when considering NMT as a drug target. *In vivo* studies, were conducted in collaboration with MMV in the SCID mouse *P. falciparum* model, which showed clearance of parasites from the infected mice after three days comparable to chloroquine with a 50mg/kg oral daily dosage. NMT inhibitors act on metabolically active parasite stages (see section 3.2). They start to kill when parasites enter schizogony and have also been shown to have an effect on metabolically active liver stages of *P. berghei* (which will also be relevant for prophylaxis and targeting dormancy in *P. vivax*) but are less active for example on gametocytes (for transmission blocking) (unpublished data in discussion with Andrew Bell and Edward Tate). Taken together data presented in this thesis as well as collected in collaboration with MMV show that NMT is a promising drug target fulfilling most of the criteria discussed in section 1.6.4, such as having a novel MoA without cross-resistance to any of the currently used antimalarial drugs and being active against both liver and sexual blood stages. However, not all criteria are fulfilled since NMT inhibitors are not able to target the *P. vivax* hypnozoite or the sexual

gametocytes since both are metabolically dormant stages, nor are they able to be administered as a single-dose cure yet.

Compared to other antimalarial drugs currently on the market, no other approved inhibitor targets myristoylation or other PTM, creating a novel MoA for NMT inhibition. To further study the phenotype underlying NMT inhibition and to study the biology of its substrates, it is important to examine the function of proteins upstream and downstream of NMT, and the phenotype resulting from their ablation. Although many *Plasmodium* NMT substrates likely remain to be discovered, it is clear that several of those already identified experimentally have an essential role in the erythrocytic cycle of the malaria parasite. Further experimental evidence to support this was provided in this thesis by using GAP45 as an example to inhibited myristoylation in isolation on one specific substrate compared to the introduction of the pleiotropic phenotype through NMT inhibition. *P. falciparum* parasites only expressing GAP45[G2A], are not able to start their erythrocyte life cycle since the invasion machinery consisting of the actinomyosin motor localised between the IMC and the PPM is not fully functional without the N-terminal myristylation of GAP45 (see Chapter 4). Compared to this very specific phenotype following the lack of myristoylation on GAP45, NMT inhibition induces a pleiotropic phenotype involving all of its substrates. Some known antimalarial agents kill the parasite by inhibiting targets involved directly or indirectly in protein synthesis (e.g. DDD107498 (Baragaña et al. 2015)), often in the last 24 – 48 h of the erythrocytic cycle. This timing coincides with that of NMT inhibitors presumably because myristoylation is a co-translational process, and may affect a similar spectrum of essential proteins as protein synthesis inhibitors acting upstream of NMT. Furthermore, there is a significant decrease in abundance of DNA-associated proteins in NMT inhibited parasites, connecting NMT inhibition and DNA replication and/or protein synthesis machinery. Inhibition of NMT also affects the stability of some NMT substrates which decrease in abundance and contribute to the pleiotropic consequences of parasites not being able to complete the life cycle and egress even after only an eleven-hour inhibition (see Chapter 3).

Going forward, although extensively validated as a drug target, NMT drug development needs further work to improve the selectivity over the human NMT

enzyme, as well as improving the pharmacokinetic profile of the inhibitors used such as their half-life, clearance and bioavailability. Some of this can hopefully be addressed with a new NMT drug series being developed at GSK as well as at Imperial College. The series at both GSK and Imperial College have different binding modes compared to the chemical series around IMP-1002. But they both still target the substrate binding pocket of NMT. It would be important to determine whether compounds in those inhibitor series can overcome the G386E-mediated resistance without compromising selectivity of the human NMT, as it is the case for DDD85646 (see Chapter 5). In addition, it would be interesting to see if other possible resistance mechanisms can be selected for with the newly developed compounds in wild type parasites but also in the already established G386E mutant line. Furthermore, it would be interesting to see what resistance will be selected for when two NMT inhibitors (e.g. IMP-1002 and DDD856464) are used in combination, as this would be a likely scenario taking NMT inhibitors into pre-clinical trials.

There is much opportunity to apply the tools used in this study to extend the knowledge on NMT substrates by labelling with YnMyr earlier in the erythrocytic stage of the *P. falciparum* cycle as well as using the rodent parasite *P. berghei* to identify *N*-myristoylated substrates in different life cycle stages such as the liver and mosquito stages. Expanding on the identification of NMT substrates in ring and trophozoite stages as well as proteins exported during the erythrocyte life cycle could shine more light on some interesting biology such as alternative protein secretion in the case of NMT substrate AK2 (Thavayogarajah et al. 2015) and Rab5b (Ebine et al. 2016) which are both thought to be exported across the PPM to the TVN facilitated through myristoylation via an uncharacterized membrane-trafficking pathway. It is possible that myristoylation also plays a role in transporting other proteins across the PPM to the PVM or even to the RBC membrane. For example, some PfEMP1 proteins, localized at the RBC membrane, are predicted to be myristoylated (Wright et al. 2014), leaving room for further investigation.

To allow the study of on-target engagement of NMT inhibitors *in vivo*, a recently developed assay can be utilized on the basis of the enzyme Sortase A (Goya Grocin et al. 2019). *Staphylococcus aureus* Sortase A (SrtA) is a transpeptidase that can be used to attach a capture reagent to a free N-terminal glycine. The enzyme cleaves

between threonine and glycine residues of a recognition motif of its substrate and then catalyses the formation of an amide bond between the carboxyl-group of the threonine and the amino-group of the cell-wall peptidoglycan. If a peptide is provided which contains the recognition sequence as well as capture reagents it will be ligated onto a free N-terminal glycine by SrtA (Goya Grocin et al. 2019). Inhibition with the NMT inhibitor will generate free N-terminal glycine as no myristic acid will be attached, allowing for the attachment of the capture reagent through SrtA. This approach is complementary to YnMyr metabolic tagging, as the loss of signal seen with YnMyr with an inhibitor present mirrors the increase in signal of newly synthesised proteins exposing an N-terminal glycine through the SrtA assay. This strategy can overcome some of the limitations of metabolic tagging, as it can be used in samples that are not metabolically active such as cell lysates. Therefore, it also has the potential to work on cell lysates from an *in vivo* experiment to show on-target activity of the new NMT inhibitors.

Chapter 7. Appendix

7.1 Data tables

PlasmoGem essential	PlasmoGem reduced growth rate	PlasmoGem Dispensible	PiggyBac essential	PiggyBac reduced growth rate	PiggyBac Dispensible	P. falciparum KO essential
ARO (PF3D7_0414900)	A/B hydrolase S9C (PF3D7_0403800)	CDPK1 (PF3D7_0217500)	S/T kinase (PF3D7_1450000)	RING zinc (PF3D7_0215100)	ADP-R factor-like (PF3D7_0920500)	Calpain (PF3D7_1362400)
RPT2 (PF3D7_1008400)	CLPTMI-D (PF3D7_1137300)	TRP-putative (PF3D7_0601600)	A/B hydrolase S9C (PF3D7_0403800)	Cytochrome C Ox. Chaperon (PF3D7_1025600)	CPIUF (PF3D7_0913800)	GAP45 (PF3D7_1222700)
ARF1 (PF3D7_1020900)	CNB (PF3D7_1451700)	CPIUF (PF3D7_1237700)	TRP-putative (PF3D7_0601600)	PKAr (PF3D7_1223100)	G2 protein (PF3D7_0929600)	AK2 (PF3D7_0816900)
GAP45 (PF3D7_1222700)		Rab5b (PF3D7_1310600)	VPS46 (PF3D7_0906100)	TOM22 (PF3D7_0524700)	Pantothenate Kinase (PF3D7_1437400)	
S/T kinase (PF3D7_1450000)		ISP3 (PF3D7_1460600)	PM2 (PF3D7_1138500)	ADP-R factor like (PF3D7_1034700)	CNB (PF3D7_1451700)	
Calpain (PF3D7_1362400)		ISPI (PF3D7_1011000)	PM2 (PF3D7_1138500)	ADP-R factor like (PF3D7_1011000)	CDPK4 (PF3D7_0717500)	
		TOM22 (PF3D7_0524700)	VSP2 (PF3D7_1237700)	ISPI (PF3D7_1011000)	BDS-domain (PF3D7_0423200)	
		A/B hydrolase S9B (PF3D7_0805000)	ARF1 (PF3D7_1020900)	APH (PF3D7_0414600)	PPM5 (PF3D7_0810300)	
		ADP-R factor like (PF3D7_1034700)	CDPK1 (PF3D7_0217500)	ARO (PF3D7_0414900)	GRASP1 (PF3D7_1017300)	
		G2 protein (PF3D7_0929600)	AK2 (PF3D7_0816900)	RPT2 (PF3D7_1008400)	CLPTMI-D (PF3D7_1137300)	
		Pantothenate Kinase (PF3D7_1437400)	Calpain (PF3D7_1362400)	A/B hydrolase S9B (PF3D7_0805000)	GAP45 (PF3D7_1222700)	
		AK2 (PF3D7_0816900)			Rab5b (PF3D7_1310600)	

Table 7.1 Data table for Figure 1.4 C and D.
Essentiality analysis of NMT substrates based on *P. berghei* PlasmoGem screen (Bushell *et al.*, 2017 and Schwach *et al.*, 2015) and the *P. falciparum* piggyBac insertional mutagenesis screen (Zhang *et al.*, 2018).

Membrane associated	CLPTMI-D (PF3D7_1137300)	CPIUF (PF3D7_1237700)	GAP45 (PF3D7_1222700)	G2 protein (PF3D7_0928600)
Golgi	ARF1 (PF3D7_1020900)	GRASP1 (PF3D7_1017300)		
IMC	ISP3 (PF3D7_1460600)	ISP1 (PF3D7_1011000)		
proteosome	RPT2 (PF3D7_1008400)	CDPK4 (PF3D7_0717500)	PKAr (PF3D7_1223100)	
cytoplasm	CNB (PF3D7_1451700)	Cytochrome C Ox. Chaperon (PF3D7_1025600)		
mitochondrion	TOM22 (PF3D7_0524700)			
apicoplast	A/B hydrolase S9B			
unknown	TRP-putative (PF3D7_0601600)	BDS-domain (PF3D7_0423200)	RING zinc (PF3D7_0215100)	
Nucleus	Calpain (PF3D7_1362400)	PM2 (PF3D7_1138500)	PM5 (PF3D7_0810300)	
rhoptry	ARO (PF3D7_0414900)			
vacuole	VPS46 (PF3D7_0906100)			
microneme	APH (PF3D7_0414600)			
PVM	Rab5b (PF3D7_1310600)			
PM	CDPK1 (PF3D7_0217500)			

Table 7.2 Data table for Figure 1.4 B.

NMT substrates and their putative localisation

Hydrolase activity (GTPase, alpha/beta hydrolase)	Proteolysis	Phosphatase activity	Kinase activity	Transport/Mot ility/Stability	Unknown
A/B hydrolase S9B (PF3D7_0805000)	Calpain (PF3D7_1362400)	PPM2 (PF3D7_1138500)	S/T kinase (PF3D7_1450000)	TOM22 (PF3D7_0524700)	RING zinc (PF3D7_0215100)
A/B hydrolase S9C (PF3D7_0403800)		PPM5 (PF3D7_0810300)	Pantothenate Kinase (PF3D7_1437400)	GRASP1 (PF3D7_1017300)	CP/UF (PF3D7_0913800)
RPT2 (PF3D7_1008400)		CNB (PF3D7_1451700)	PKAr (PF3D7_1223100)	ISP3 (PF3D7_1460600)	CP/UF (PF3D7_1237700)
ADP-R factor-like (PF3D7_0920500)			AK2 (PF3D7_0816900)	ISP1 (PF3D7_1011000)	TRP-putative (PF3D7_0601600)
ARF1 (PF3D7_1020900)			CDPK1 (PF3D7_0217500)	GAP45 (PF3D7_1222700)	APH (PF3D7_0414600)
ADP-R factor-like (PF3D7_1034700)			CDPK4 (PF3D7_0717500)	ARO (PF3D7_0414900)	BDS-domain (PF3D7_0423200)
Rab5b (PF3D7_1310600)				VPS46 (PF3D7_0906100)	CLPTM1-D (PF3D7_1137300)
				VSP2 (PF3D7_0816200)	
				G2 protein (PF3D7_0929600)	
				Cytochrome C Ox. Chaperon (PF3D7_1025600)	
				ADP-R factor-like (PF3D7_0920500)	
				ARF1 (PF3D7_1020900)	
				ADP-R factor-like (PF3D7_1034700)	
				Rab5b (PF3D7_1310600)	

Table 7.3 Data table for figure 1.4 A.

NMT substrates and their putative function. Proteins with the same colour have been allocated to two groups based on their putative function

Protein ID	Protein name	Modified peptide in DMSO sample	Modified peptide IMP-1002 sample	NMT substrate (Wright <i>et al.</i> , 2014)	Modified peptide found by Wright <i>et al.</i> , 2014	Predicted NMT substrate (Wright <i>et al.</i> , 2014)
PF3D7_1011000	ISP1	Y	Y	Y	Y	Y
PF3D7_1460600	ISP3	Y	Y	Y	Y	Y
PF3D7_0920500	ADP-ribosylation factor, putative	Y		Y	Y	Y
PF3D7_0217500	CDPK1	Y		Y	Y	Y
PF3D7_1020900	ARF1	Y	Y	Y	Y	Y
PF3D7_1451700	calcineurin subunit B (CNB)	Y		Y	Y	Y
PF3D7_1237700	conserved protein, unknown function	Y	Y	Y	Y	Y
PF3D7_0414900	ARO	Y		Y	Y	Y
PF3D7_0810300	PPM5 protein phosphatase PPM5, putative	Y	Y	Y	Y	Y
PF3D7_0631000	tetratricopeptide repeat protein, putative	Y		Y	Y	Y
PF3D7_0601600						
PF3D7_0816200	vacuolar protein sorting-associated protein 2 (VPS2)	Y		Y		Y
PF3D7_0414600	acylated pleckstrin-homology domain-containing protein, putative (APH)	Y		Y		Y
PF3D7_1309200	metal-dependent protein phosphatases 6	Y	Y			Y
PF3D7_0523000	multidrug resistance protein 1 (MDR1)	Y				Y

green = modified peptide not identified by wright *et al.*, 2014 but classified as NMT substrates due to response to base treatment

red = newly NMT substrates based on identified modified peptide - but previously predicted

Table 7.4 YnMyr modified peptides of proteomic pull-down experiment.

Proteins where a modified peptide has been identified in the YnMyr pull down experiment (see Figure 3.6). The table indicates if the peptide was found in the DMSO treated sample, or in the IMP-1002 (IMP) treated sample or both. The dataset from Wright *et al.*, 2014 was analysed to compare if a protein has previously been classified as an NMT substrate, and if this was based on the identification of the modified peptide, or prediction (or no response to NaOH treatment).

Gene IDs	description	sub-category	category	-Log Student's T-test p-value IMP_DMSO	Student's T-test Difference (-log ₁₀)
PF3D7_1401100	DnaJ protein	chaperones	Chaperones	3.87	-0.57
PF3D7_1119700	conserved Plasmodium protein	conserved protein	conserved protein	1.56	-1.71
PF3D7_1239800	conserved Plasmodium protein	conserved protein	conserved protein	3.19	-1.68
PF3D7_1248700	conserved Plasmodium protein	conserved protein	conserved protein	3.46	-0.96
PF3D7_1126700	autophagy-related protein 23	conserved protein	conserved protein	3.38	-0.88
PF3D7_0813300	conserved Plasmodium protein	conserved protein	conserved protein	2.67	-0.88
PF3D7_0214700	conserved Plasmodium protein	conserved protein	conserved protein	2.10	-0.82
PF3D7_1035900	probable protein	conserved protein	conserved protein	4.57	-0.82
PF3D7_0410800	conserved Plasmodium protein	conserved protein	conserved protein	2.01	-0.82
PF3D7_1241200	conserved Plasmodium protein	conserved protein	conserved protein	4.38	-0.79
PF3D7_1308400	conserved Plasmodium protein	conserved protein	conserved protein	4.06	-0.71
PF3D7_1032700	conserved Plasmodium protein	conserved protein	conserved protein	3.48	-0.64
PF3D7_1436200	conserved Plasmodium protein	conserved protein	conserved protein	2.68	-0.63
PF3D7_0407800	conserved Plasmodium protein	conserved protein	conserved protein	3.40	-0.58
PF3D7_1004500	conserved Plasmodium protein	conserved protein	conserved protein	3.13	-0.58
PF3D7_0820000	Snf2-related CBP activator	Transcription	DNA/chromatin associated	2.82	-2.73
PF3D7_0714000	histone H2B variant	Histone	DNA/chromatin associated	2.76	-2.35
PF3D7_1031200	MORN repeat-containing protein 1	"multiple membrane occupation and recognition nexus" - functions as a linker protein between certain membrane regions and the parasite's cytoskeleton in <i>T. gondii</i> - associated with a set of subcellular structures that play key roles in parasite mitosis, daughter cell formation and cytokinesis	DNA/chromatin associated	3.51	-1.98
PF3D7_0610400	histone H3	Histone	DNA/chromatin associated	2.97	-1.29
PF3D7_1008100	zinc finger protein	DNA recognition, RNA packaging, transcriptional activation, protein folding and assembly, and lipid binding.	DNA/chromatin associated	4.07	-1.22
PF3D7_0517400	FACT complex subunit SPT16	Transcription - heterodimeric protein complex that affects eukaryotic RNA polymerase II (Pol II) transcription elongation	DNA/chromatin associated	4.00	-1.20
PF3D7_1105000	histone H4	Histone	DNA/chromatin associated	3.39	-1.20
PF3D7_1212900	bromodomain protein 2	Chromatin organisation	DNA/chromatin associated	4.58	-1.05
PF3D7_1027700	centrin-3	DNA replication - centrosome duplication and separation	DNA/chromatin associated	3.18	-1.00
PF3D7_0202400	translation-enhancing factor	Translation	DNA/chromatin associated	4.27	-0.89
PF3D7_0711500	regulator of chromosome condensation	Chromatin organisation	DNA/chromatin associated	3.49	-0.87
PF3D7_1008000	histone deacetylase 2	Histone	DNA/chromatin associated	3.52	-0.84
PF3D7_0320900	histone H2A.2	Histone	DNA/chromatin associated	5.04	-0.81
PF3D7_0617900	histone H3 variant	Histone	DNA/chromatin associated	1.36	-0.72
PF3D7_1104200	chromatin remodeling protein	Chromatin organisation	DNA/chromatin associated	4.51	-0.70
PF3D7_1333700	histone H3-like centromeric protein CSE4	Histone	DNA/chromatin associated	3.30	-0.67
PF3D7_1412000	p1/s1 nuclease	DNA replication and transcription - cleaves RNA and single-stranded DNA	DNA/chromatin associated	4.73	-0.67
PF3D7_1105100	histone H2B	Histone	DNA/chromatin associated	4.71	-0.65
PF3D7_1439500	oocyst rupture protein 2	Transcriptional - essential for rupture of the oocysts in <i>P. berghei</i> - contains two histone fold domains - form heterodimer - role in transcriptional regulation (Siden-Kiamos et al., 2018)	DNA/chromatin associated	2.54	-0.65
PF3D7_1433500	DNA topoisomerase 2	DNA replication and transcription	DNA/chromatin associated	3.78	-0.64
PF3D7_1220900	heterochromatin protein 1	Chromatin organisation	DNA/chromatin associated	3.08	-0.59
PF3D7_0520400	single-stranded DNA-binding protein	DNA replication	DNA/chromatin associated	4.10	-0.59
PF3D7_0624600	SNF2 helicase	DNA replication	DNA/chromatin associated	4.97	-0.56
PF3D7_1441400	FACT complex subunit SSRP1	Transcription - heterodimeric protein complex that affects eukaryotic RNA polymerase II (Pol II) transcription elongation	DNA/chromatin associated	4.13	1.57
PF3D7_0525800	inner membrane complex protein 1g	IMC complex protein	IMC complex protein	2.54	-1.02
PF3D7_1351700	inner membrane complex protein 1f	IMC complex protein	IMC complex protein	4.97	-0.90
PF3D7_0304100	inner membrane complex protein 1e	IMC complex protein	IMC complex protein	2.45	-0.88
PF3D7_1460600	inner membrane complex sub-compartment protein 3	NMT substrate	NMT substrate	3.40	-1.37
PF3D7_0403800	alpha/beta hydrolase	NMT substrate	NMT substrate	2.33	-1.13
PF3D7_1222700	glideosome-associated protein 45	NMT substrate	NMT substrate	3.82	-0.83
PF3D7_0414900	armadillo-domain containing rhoptry protein	NMT substrate	NMT substrate	3.83	-0.71
PF3D7_0631000	tetratricopeptide repeat protein	NMT substrate	NMT substrate	4.22	-0.68
PF3D7_0217500	calcium-dependent protein kinase 1	NMT substrate	NMT substrate	3.62	0.72
PF3D7_0507500	subtilisin-like protease 1	Peptidase (SUB1-like)	Peptidase	3.22	-0.84
PF3D7_0115600	rifin	erythrocyte membrane - repetitive interspersed family protein - possible role in cytoadhesion and immune	Secreted/Exported	2.81	-2.34
PF3D7_0201900	erythrocyte membrane protein 3	erythrocyte membrane	Secreted/Exported	3.31	-1.65
PF3D7_0424600	Plasmodium exported protein (PHISTb)	erythrocyte membrane (beyond PVM)	Secreted/Exported	3.16	-1.18
PF3D7_0202000	knob-associated histidine-rich protein	erythrocyte membrane	Secreted/Exported	3.01	-0.76
PF3D7_0936800	Plasmodium exported protein (PHISTc)	erythrocyte membrane	Secreted/Exported	2.53	-0.71
PF3D7_0113000	glutamic acid-rich protein	erythrocyte membrane	Secreted/Exported	4.34	-0.70
PF3D7_1228600	merozoite surface protein 9	merozoite surface	Secreted/Exported	6.00	-0.68
PF3D7_0219800	Plasmodium exported protein (PHISTc)	erythrocyte membrane (beyond PVM)	Secreted/Exported	4.30	-0.64
PF3D7_0401600	rifin	erythrocyte membrane - repetitive interspersed family protein - possible role in cytoadhesion and immune	Secreted/Exported	2.49	-0.62
PF3D7_0500800	mature parasite-infected erythrocyte surface antigen	erythrocyte membrane	Secreted/Exported	3.51	-0.58
PF3D7_0908000	plasma membrane protein 1	plasma membrane	Secreted/Exported	5.00	-0.57
PF3D7_0532400	lysine-rich membrane-associated PHISTb protein	erythrocyte membrane	Secreted/Exported	3.97	0.74
PF3D7_1236000	vesicle transport v-SNARE protein VT11	transport vesicles during budding	Transport	3.03	-0.66

Table 7.5 Data table for significantly downregulated proteins from IMP-1002 whole proteomic analysis.

For larger table please see provided excel spread sheet ("Appendix Whole proteomics +Sapo"). The GO term analysis function on PlasmoDB was used to classify the proteins in groups based on function and location. Related to Figure 3.8 and 3.9.

Gene ID	Protein abbreviation	Product/Description	Identified by Wright et al., 2014	Modified peptide present in YmYr pull down	Significant in YmYr pull down	YmYr Log ₂ Student's T-test Difference - whole proteomics value (FDR 0.02)	YmYr Log ₂ IMP, DMSO	Whole proteomics - whole proteomics Difference - log IMP, DMSO	Significant in whole proteomics test (p-value FDR 0.02)	Whole proteomics - whole proteomics test (p-value FDR 0.02)	N-terminal modification	Function	Location/components
PF3D7_0215100	RING zinc	RING zinc finger protein, putative	+								N-myristoylation	not known	not known
PF3D7_0217500	CDRK1	calcium-dependent protein kinase 1	+	+	+	3.0	-2.4	-0.8	+	4.6	N-myristoylation and N-palmitoylation	ATP binding, calcium ion binding, protein kinase activity, calcium-dependent protein kinase activity, entry into host cell, protein serine/threonine kinase activity, entry into host cell, protein tyrosine kinase activity, protein phosphorylation	membrane, plasma membrane, symbiont-containing vacuole, symbiont-containing vacuole membrane
PF3D7_0321400	Putative Kinase	protein kinase, putative		+	+	4.2	-1.4	-0.1		0.5	N-myristoylation	entry into host cell, protein serine/threonine kinase activity, protein phosphorylation	vacuole membrane
PF3D7_0403800	AB hydrolase (SBC)	alpha-beta hydrolase, putative (SBC)	+	+	+	2.0	-3.5	-1.0	+	3.5	N-myristoylation	hydrolase activity	nuclear periphery, nucleus
PF3D7_0414600	APH	acylated pleckstrin-homology domain-containing protein, putative	+								N-myristoylation	not known	microneme
PF3D7_0414900	ARO	aromatase-like domain containing thiolase protein	+	+	+	3.7	-3.0	-0.9	+	4.3	N-myristoylation and 2x N-palmitoylation	localization of mitochondria, binding, DNA binding, protein transport	anchored component of membrane, cytosol, Golgi apparatus, apical
PF3D7_0423200	BSD-domain	BSD-domain protein, putative	+								N-myristoylation	not known	not known
PF3D7_0524700	TOM22	mitochondrial import receptor subunit TOM22, putative	+	+							N-myristoylation	protein targeting to mitochondrion, intracellular protein transport	mitochondrial outer membrane translocase complex, mitochondrion
PF3D7_0601600	TRP-putative	tetraspanin-like protein, putative	+	+	+	2.4	-2.6	-1.2	+	4.1	N-myristoylation	protein binding	no predictions
PF3D7_0615700	CPUF	conserved protein, unknown function	+	+	+	4.0	-1.6	-0.1		3.1	N-myristoylation	protein binding	no predictions
PF3D7_0631000	TRP-putative	tetraspanin-like repeat protein, putative	+	+	+	2.4	-2.6	-1.2	+	4.1	N-myristoylation	protein binding	no predictions
PF3D7_0717500	CDPK4	calcium-dependent protein kinase 4	+								N-myristoylation	ATP binding, calcium ion binding, calcium-dependent protein kinase activity, catalytic activity, DNA replication, mitotic spindle assembly, pre-replicative complex assembly, protein phosphorylation	mitochondrion maintenance protein complex (RCLN)
PF3D7_0805000	AB hydrolase (SBB)	alpha-beta hydrolase, putative (SBB)		+							N-myristoylation	putative Serine protease	Apicoplast
PF3D7_0810300	PMK5	metal-dependent protein phosphatase 5	+	+							N-myristoylation and N-palmitoylation	catalytic activity, protein serine/threonine phosphatase activity, cation binding	Nucleus
PF3D7_0816200	VSP2	vacuolar protein sorting-associated protein 2, putative	+	+	+	2.2	-2.1	-0.1		1.7	N-myristoylation and N-palmitoylation	protein transport, vacuolar transport	no predictions
PF3D7_0906100	VPS46	vacuolar protein sorting-associated protein 46, putative	+								N-myristoylation	protein and vacuolar transport	Vacuole
PF3D7_0913800	CPUF	conserved Plasmodium protein, unknown function	+								N-myristoylation	not known	Cytoplasm
PF3D7_0920500	ADP-R factor-like	ADP-ribosylation factor, putative	+	+	+	3.8	-2.1	0.0		0.1	N-myristoylation and N-palmitoylation	small GTPase mediated signal transduction, intracellular, GTP binding	intracellular - no predictions on location
PF3D7_0928600	G2 protein	G2 protein, putative	+								N-myristoylation	SPN (subpellicular network)	pellicle
PF3D7_1008400	RPT2	26S protease regulatory subunit 4, putative	+	+	+	2.7	-2.6	0.2		3.0	N-myristoylation	ATP binding, hydrolase activity, protein catalytic process, proteasome regulatory subunit activity, ubiquitin-dependent protein catabolic process, ubiquitin-dependent protein catabolic process	cytoplasm, proteasome regulatory particle, base subcomplex
PF3D7_1011000	ISP1	inner membrane complex sub-compartment protein 1	+	+	+						N-myristoylation and 2x N-palmitoylation	regulating IMC formation, protein trafficking, gliding motility and invasion	apical part of cell, cytosol, inner membrane pellicle complex
PF3D7_1017300	GRASP1	golgi re-assembly stacking protein 1, golgi re-assembly stackin	+								N-myristoylation	Golgi and organelle biogenesis, protein binding, protein dimerization activity, golgi organization	Golgi apparatus, cis-Golgi network
PF3D7_1024900	ARF1	ADP-ribosylation factor	+	+	+	2.7	-1.4	-0.2		3.9	N-myristoylation and N-palmitoylation	GTP-binding protein - ER-Golgi system, membrane trafficking, intracellular protein transport, protein ADP-ribosylation	Golgi apparatus, cis-Golgi network
PF3D7_1025600	Cytochrome C Ox. Chaperone	cytochrome c oxidase copper chaperone, putative	+								N-myristoylation	oxidoreductase activity, copper ion binding and transport	mitochondrial intermembrane space
PF3D7_1034700	ADP-R factor like	ADP-ribosylation factor, putative	+	+	+						N-myristoylation	small GTPase mediated signal transduction, intracellular protein transport, GTP binding	intracellular - no predictions on location
PF3D7_1137300	CLPTM1-4	CLPTM1 domain-containing protein, putative	+	+	+	3.8	-1.5	0.0		0.1	N-myristoylation and N-palmitoylation	nothing predicted	integral component of membrane
PF3D7_1138500	PMK2	metal-dependent protein phosphatase 2	+								N-myristoylation	catalytic activity, protein serine/threonine phosphatase activity, cation binding, protein phosphorylation	nucleus
PF3D7_1222700	GAP45	glucocorticoid-associated protein 45	+	+	+	3.9	-3.1	-0.6	+	3.5	N-myristoylation and 2x C-palmitoylation	regulating and stabilizing the actomyosin motor complex, entry into host cell (invasion)	extrinsic component of membrane, host cell membrane, inner membrane pellicle complex
PF3D7_1225100	PMK4	CAMP-dependent protein kinase regulatory subunit	+	+							N-myristoylation and N-palmitoylation	regulation of the cAMP-dependent protein kinase activity, protein dimerization, protein stages and gametogenesis, protein trafficking, regulation of protein phosphorylation	CAMP-dependent protein kinase complex
PF3D7_1237700	CPUF	conserved protein, unknown function	+	+	+	4.9	-1.4	0.0		0.3	N-myristoylation and N-palmitoylation	nothing predicted	integral component of membrane, cell surface
PF3D7_1316600	Rab5b	ras-related protein Rab-5b	+	+	+						N-myristoylation and N-palmitoylation	protein transport by the Sec complex, small GTPase, regulation of nucleoside-mediated transport	food vacuole, plasma membrane, vacuole, vacuole membrane, vacuole membrane
PF3D7_1437400	Pantothrate Kinase	pantothrate kinase 2, putative	+								N-myristoylation and N-palmitoylation	panthoate kinase activity, ATP binding, protein phosphorylation, transferase activity, ATP binding, protein kinase activity	panthoate kinase activity, ATP binding, protein phosphorylation, transferase activity, ATP binding, protein kinase activity
PF3D7_1450000	S/T Kinase	serine/threonine protein kinase, putative	+	+	+	2.2	-2.5	-0.5		3.1	N-myristoylation	calcium-dependent protein serine/threonine phosphatase regulator activity, entry into host cell, protein phosphorylation	cytoplasm, calcineurin complex
PF3D7_1451700	ONB	calcineurin subunit B	+	+	+						N-myristoylation	calcineurin complex	cytoplasm, calcineurin complex
PF3D7_1460600	ISP3	inner membrane complex sub-compartment protein 3	+	+	+	2.2	-2.3	-0.8	+	3.2	N-myristoylation and 2x N-palmitoylation	regulating IMC formation, protein trafficking, gliding motility and invasion	cell periphery, inner membrane pellicle complex

Table 7.6 NMT substrates analysis 1

For larger table please see the provided excel file ("NMTs table 1")

<i>P. falciparum</i> Gene ID	<i>P. berghei</i> Gene ID	Protein abbreviation	Significant in pull down	Significant in whole proteomics (saponin lysed)	KO in <i>P. falciparum</i> / <i>P. berghei</i> asexual stages	Essential for asexual cycle (piggyBac / Plasmogem)	Plasmogem Gomes et al., 2015, Schwach et al., 2015 Relative Growth rate	Plasmogem phenotype	<i>P. falciparum</i> 3D7 piggyBac insertion mutagenesis Zhang et al., 2108 - mutagenesis index score 1=dispensable, 0 = essential	<i>P. falciparum</i> 3D7 piggyBac insertion mutagenesis - mutant fitness score -4 = essential, 4 = dispensable
PF3D7_1450000	PBANKA_1313700	S/T kinase	+		no	essential (<i>P. falciparum</i>)			0.13	-2.93
PF3D7_0403800	PBANKA_1001500	A/B hydrolase (S9C)	+	+	no	essential (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.77	Slow	0.13	-2.95
PF3D7_0631000	PBANKA_1129800	TRP-putative	+	+	no	essential (<i>P. falciparum</i>) dispensable (<i>P. berghei</i>)	1.07	Dispensable	0.13	-2.94
PF3D7_0906100	PBANKA_0415200	VPS46			no	essential (<i>P. falciparum</i>)			0.14	0.00
PF3D7_0619700	PBANKA_1118900	CP/UF	+		no	essential (<i>P. falciparum</i>)			0.14	-3.01
PF3D7_1138500	PBANKA_0910200	PPM2			no	essential (<i>P. falciparum</i>)			0.19	-2.80
PF3D7_1237700	PBANKA_1452300	CP/UF	+		no	essential (<i>P. falciparum</i>)			0.19	-3.37
PF3D7_0816200	PBANKA_0714600	VSP2	+		no	essential (<i>P. falciparum</i>)			0.24	-2.94
PF3D7_1020900	PBANKA_0505100	ARF1	+		no	essential (<i>P. falciparum</i>) essential (<i>P. berghei</i>)	0.07	Essential	0.25	-3.26
PF3D7_0217500	PBANKA_0314200	CDPK1	+	+	yes <i>P. berghei</i> Tewari et al., 2009 and <i>P. falciparum</i> Doering et al., 2007 (dispensable / unsuccessful KO)	essential (<i>P. falciparum</i>) slow or dispensable (<i>P. berghei</i>)	0.79	Slow	0.27	-2.63
PF3D7_0321400	PBANKA_1217100	Putative Kinase	+		no	slow (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.85	Slow	0.31	-2.59
PF3D7_0215100	PBANKA_0311900	RING zinc			no	slow (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.79	Slow	0.36	0.00
PF3D7_1025600	PBANKA_0509800	Cytochrome C Ox. Chaperon			no	slow (<i>P. falciparum</i>)			0.37	-3.61
PF3D7_1223100	PBANKA_1438000	PKAr			no	slow (<i>P. falciparum</i>)			0.40	-2.62
PF3D7_0524700	PBANKA_1239500	TOM22			no	slow (<i>P. falciparum</i>) essential (<i>P. berghei</i>)	0.05	Essential	0.47	-2.53
PF3D7_1034700	PBANKA_0518500	ADP-R factor like			no	slow (<i>P. falciparum</i>) dispensable (<i>P. berghei</i>)	1.00	Dispensable	0.49	-2.90
PF3D7_1011000	PBANKA_1209400	ISP1			yes <i>P. berghei</i> (essential)	slow (<i>P. falciparum</i>) - essential <i>P. berghei</i> Knock out (Poulin et al., 2013)			0.51	0.00
PF3D7_1460600	PBANKA_1324300	ISP3	+	+	yes <i>P. berghei</i> (dispensable)	slow (<i>P. falciparum</i>) - dispensable <i>P. berghei</i> Knock out (Poulin et al., 2013)			0.57	-2.26
PF3D7_0414600	PBANKA_0716600	APH			no	slow (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.79	Slow	0.68	-2.21
PF3D7_0414900	PBANKA_0716900	ARO	+	+	no	slow (<i>P. falciparum</i>)			0.68	-2.21
PF3D7_1008400	PBANKA_1206600	RPT2	+		no	slow (<i>P. falciparum</i>) dispensable (<i>P. berghei</i>)	1.01	Dispensable	0.75	-2.43
PF3D7_0805000	PBANKA_1225600	A/B hydrolase (S9B)			no	slow (<i>P. falciparum</i>) linkly not essential (<i>P. berghei</i>)	1.03	Dispensable	0.75	-3.00
PF3D7_0920500	PBANKA_0821400	ADP-R factor-like	+		no	dispensable (<i>P. falciparum</i>)			0.82	-2.01
PF3D7_0913800	PBANKA_0814800	CP/UF			no	dispensable (<i>P. falciparum</i>)			0.85	0.00
PF3D7_0929600	PBANKA_0830400	G2 protein			no	dispensable (<i>P. falciparum</i>)			0.90	-2.42
PF3D7_1437400	PBANKA_0611400	Pantothenate Kinase			no	dispensable (<i>P. falciparum</i>) dispensable (<i>P. berghei</i>)	0.91	Dispensable	0.92	-2.93
PF3D7_1451700	PBANKA_1315400	CNB			no	dispensable (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.18	Slow	1.00	0.00
PF3D7_0717500	PBANKA_0615200	CDPK4			no	dispensable (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.86	slow	1.00	-2.79
PF3D7_0423200	PBANKA_0523600	BDS-domain			no	dispensable (<i>P. falciparum</i>)			1.00	-1.35
PF3D7_0601600	PBANKA_1129800	TRP-putative	+	+	no	dispensable (<i>P. falciparum</i>) dispensable(<i>P. berghei</i>)	1.07	Dispensable	1.00	-2.19
PF3D7_0810300	PBANKA_1427200	PPM5			no	dispensable (<i>P. falciparum</i>) dispensable(<i>P. berghei</i>)	1.11	Dispensable	1.00	-2.05
PF3D7_1017300	PBANKA_0501600	GRASP1			no	dispensable (<i>P. falciparum</i>)			1.00	0.85
PF3D7_1137300	PBANKA_0911400	CLPTM1-D	+		no	dispensable (<i>P. falciparum</i>) slow (<i>P. berghei</i>)	0.73	Slow	1.00	-2.34
PF3D7_1222700	PBANKA_1437600	Gap45	+	+	yes <i>P. falciparum</i> (essential)	dispensable (<i>P. falciparum</i>) PiggyBac - essential <i>P. falciparum</i> Knock out			1.00	-1.81
PF3D7_1310600	PBANKA_1409100	RabSb	+		no	dispensable (<i>P. falciparum</i>) essential (<i>P. berghei</i>)	0.23	Essential	1.00	2.77

Table 7.7 NMT substrate analysis 2.

For bigger table please see provided excel file (“NMTs table 2”).

7.2 Crystallographic appendix

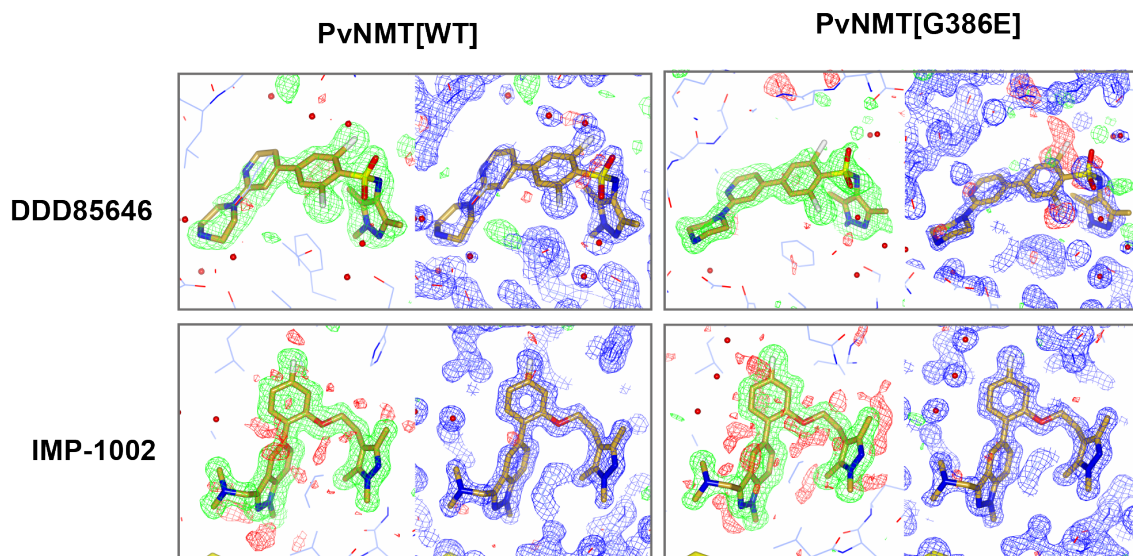


Figure 7.1 Omit map for DDD85646 and IMP-1002 bound to PvNMT[WT] and PvNMT[G386E].

For each panel, FO-FC (contoured at 3.0 sigma) density for fully refined structure with inhibitor atoms removed is shown on the left. For clarity, inhibitor stick representation is overlaid. For each panel, on the right is shown 2FO-FC (contoured at 1.0 sigma) and FO-FC (contoured at 3.0 sigma) density from fully refined structure including inhibitor atoms. Graphic was generated by Stephen Mayclin at Seattle Structural Genomics Center for Infectious Disease (SSGCID) in Seattle and Beryllium Discovery Corp. on Bainbridge Island. Related to Figure 5.10 and 5.12.

	PvNMT[G386E] Apo	PvNMT[WT] + IMP1002	PvNMT[G386E] + IMP1002	PvNMT[G386E] + DDD85646
Wavelength		0.9787	0.9787	0.9787
Resolution range		50 - 1.5 (1.54 - 1.50)	50 - 1.55 (1.59 - 1.55)	50 - 1.55 (1.59 - 1.55)
Space group		P 21 21 21	P 21 21 21	P 21 21 21
Unit cell		57.56 119.11 176.08 90 90 90	57.44 121.19 178.25 90 90 90	57.5 121.25 177.93 90 90 90
Total reflections		1120216 (63971)	1064299 (76554)	1116596 (81282)
Unique reflections		193711 (14201)	176323 (12704)	180669 (13216)
Multiplicity		5.8 (4.5)	6.0 (6.0)	6.2 (6.2)
Completeness (%)		99.9 (99.9)	97.6 (96.2)	100.0 (100.0)
Mean I/sigma(I)		11.91 (2.62)	12.78 (3.18)	18.81 (3.40)
Wilson B-factor		12.23	11.32	12.57
R-merge		0.090 (0.462)	0.094 (0.544)	0.066 (0.537)
R-meas		0.099 (0.523)	0.102 (0.595)	0.073 (0.586)
CC1/2		0.997 (0.822)	0.997 (0.838)	0.999 (0.887)
Reflections used in refinement		193683 (19198)	176228 (17160)	180657 (17875)
Reflections used for R-free		2060 (195)	1978 (196)	2028 (207)
R-work		0.1549 (0.2129)	0.1526 (0.2258)	0.1450 (0.1861)
R-free		0.1839 (0.2363)	0.1780 (0.2812)	0.1657 (0.2327)
CC(work)		0.971 (0.892)	0.974 (0.884)	0.975 (0.935)
CC(free)		0.964 (0.827)	0.969 (0.812)	0.972 (0.927)
Number of non-hydrogen atoms		11841	11918	11941
macromolecules		9687	9752	9707
ligands		325	314	314
solvent		1829	1852	1920
Protein residues		1144	1144	1143
RMS(bonds)		0.005	0.006	0.006
RMS(angles)		0.79	0.82	0.84
Ramachandran favored (%)		97.71	97.8	97.71
Ramachandran allowed (%)		2.29	2.2	2.29
Ramachandran outliers (%)		0	0	0
Rotamer outliers (%)		0.55	0.55	0.28
Clashscore		0.9	1.55	2.56
Average B-factor		17.1	16.06	17.82
macromolecules		14.98	13.85	15.32
ligands		14.28	11.87	19.58
solvent		28.85	28.38	30.16
Number of TLS groups		17	16	22

Table 7.8 Data collection and refinement statistics of crystallographic data.
Related to Figure 5.10 and 5.12.

7.3 Papers published during the course of this PhD thesis.

Please attached appendix / separate pdf.

7.4 Permission documents.

Permission documents to republish all the third party copyrighted work in this thesis. Please attached appendix / separate pdf.

Reference List

- Absalon, S. et al., 2018. Calcium-Dependent Protein Kinase 5 Is Required for Release of Egress-Specific Organelles in *Plasmodium falciparum*. L. H. Miller, ed. *mBio*, 9(1), p.411.
- Achan, J. et al., 2011. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malaria Journal*, 10(1), p.144.
- Ackerman, H. et al., 2005. A comparison of case-control and family-based association methods: the example of sickle-cell and malaria. *Annals of human genetics*, 69(Pt 5), pp.559–565.
- Adams, P.D. et al., 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta crystallographica. Section D, Biological crystallography*, 66(Pt 2), pp.213–221.
- Alam, M.M. et al., 2015. Phosphoproteomics reveals malaria parasite Protein Kinase G as a signalling hub regulating egress and invasion. *Nature communications*, 6(1), p.7285.
- Alexander, D.L. et al., 2005. Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. *PLoS pathogens*, 1(2), p.e17.
- Alonso, A.M. et al., 2019. Exploring protein myristoylation in *Toxoplasma gondii*. *Experimental Parasitology*, 203, pp.8–18.
- Alving, A.S. et al., 1956. Enzymatic deficiency in primaquine-sensitive erythrocytes. *Science*, 124(3220), pp.484–485.
- Amino, R. et al., 2008. Host Cell Traversal Is Important for Progression of the Malaria Parasite through the Dermis to the Liver. *Cell Host and Microbe*, 3(2), pp.88–96.
- Amino, R. et al., 2006. Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nature medicine*, 12(2), pp.220–224.
- Andenmatten, N. et al., 2013. Conditional genome engineering in *Toxoplasma gondii* uncovers alternative invasion mechanisms. *Nature Methods*, 10(2), pp.125–127.
- Arama, C. & Troye-Blomberg, M., 2014. The path of malaria vaccine development: challenges and perspectives. *Journal of Internal Medicine*, 275(5), pp.456–466.
- Aravind, L. et al., 2003. Plasmodium Biology: Genomic Gleanings. *Cell*, 115(7), pp.771–785.
- Ariey, F. et al., 2014. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature*, 505(7481), pp.50–55.
- Ashley, E.A. & Phyo, A.P., 2018. Drugs in Development for Malaria. *Drugs*, 78(9), pp.861–879.
- Askling, H.H. et al., 2012. Management of imported malaria in Europe. *Malaria Journal*, 11(1), p.328.
- Baer, K. et al., 2007. Release of Hepatic *Plasmodium yoelii* Merozoites into the Pulmonary Microvasculature. *PLoS pathogens*, 3(11), p.e171.
- Bansal, A. et al., 2013. Characterization of *Plasmodium falciparum* calcium-dependent protein kinase 1 (PfCDPK1) and its role in microneme secretion during erythrocyte invasion. *Journal of Biological Chemistry*, 288(3), pp.1590–1602.
- Bantscheff, M. et al., 2007. Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry*, 389(4), pp.1017–1031.

- Baragaña, B. et al., 2015. A novel multiple-stage antimalarial agent that inhibits protein synthesis. *Nature*, 522(7556), pp.315–320.
- Battle, K.E. et al., 2014. Geographical variation in *Plasmodium vivax* relapse. *Malaria Journal*, 13(1), p.144.
- Baum, E. et al., 2016. Common asymptomatic and submicroscopic malaria infections in Western Thailand revealed in longitudinal molecular and serological studies: a challenge to malaria elimination. *Malaria Journal*, 15(1), p.333.
- Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T.-W., Green, J.L., Holder, A. & Cowman, A.F., 2006a. A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *Journal of Biological Chemistry*, 281(8), pp.5197–5208.
- Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T.-W., Green, J.L., Holder, A. & Cowman, A.F., 2006b. A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *Journal of Biological Chemistry*, 281(8), pp.5197–5208.
- Beeson, J.G. et al., 2019. Challenges and strategies for developing efficacious and long-lasting malaria vaccines. *Science translational medicine*, 11(474), p.eaau1458.
- Bell, A.S., 2016. *N-Myristoyltransferase as a Drug Target: A (Chemical) Space Odyssey - PhD. Thesis*. Imperial College London.
- Bell, A.S. et al., 2012. Selective Inhibitors of Protozoan Protein N-myristoyltransferases as Starting Points for Tropical Disease Medicinal Chemistry Programs M. P. Pollastri, ed. *PLoS neglected tropical diseases*, 6(4), p.e1625.
- Berliner, R.W., Earle, D.P. & of, J.T.T.J., 1948. Studies on the chemotherapy of the human malaras. VI. The physiological disposition, antimalarial activity, and toxicity of several derivatives of 4-aminoquinoline. *Am Soc Clin Investig*, 27(3 Pt 2), pp.98–107.
- Berthiaume, L. & Resh, M.D., 1995. Biochemical characterization of a palmitoyl acyltransferase activity that palmitoylates myristoylated proteins. *Journal of Biological Chemistry*, 270(38), pp.22399–22405.
- Bhatnagar, R.S. et al., 1994. Isothermal titration calorimetric studies of *Saccharomyces cerevisiae* myristoyl-CoA:protein N-myristoyltransferase. Determinants of binding energy and catalytic discrimination among acyl-CoA and peptide ligands. *Journal of Biological Chemistry*, 269(15), pp.11045–11053.
- Bhatnagar, R.S. et al., 1997. Titration calorimetric analysis of AcylCoA recognition by myristoylCoA:protein N-myristoyltransferase. *Biochemistry*, 36(22), pp.6700–6708.
- Bhatt, S. et al., 2015. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*, 526(7572), pp.207–211.
- Bhattacharjee, S. et al., 2018. Remodeling of the malaria parasite and host human red cell by vesicle amplification that induces artemisinin resistance. *Blood*, 131(11), pp.1234–1247.
- Biagini, G.A. et al., 2012. Generation of quinolone antimalarials targeting the *Plasmodium falciparum* mitochondrial respiratory chain for the treatment and

- prophylaxis of malaria. *Proceedings of the National Academy of Sciences*, 109(21), pp.8298–8303.
- Billker, O. et al., 2004. Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell*, 117(4), pp.503–514.
- Billker, O. et al., 1998. Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature*, 392(6673), pp.289–292.
- Billker, O. et al., 1997. The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of *Plasmodium berghei* in vitro. *Parasitology*, 115(1), pp.1–7.
- Blasco, B., Leroy, D. & Fidock, D.A., 2017. Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. *Nature medicine*, 23(8), pp.917–928.
- Boisson, B., Giglione, C. & Meinnel, T., 2003. Unexpected protein families including cell defense components feature in the N-myristoylome of a higher eukaryote. *Journal of Biological Chemistry*, 278(44), pp.43418–43429.
- Bologna, G. et al., 2004. N-Terminal myristoylation predictions by ensembles of neural networks. *Proteomics*, 4(6), pp.1626–1632.
- Boman, A.L. & Kahn, R.A., 1995. Arf proteins: the membrane traffic police? *Trends in biochemical sciences*, 20(4), pp.147–150.
- Bousema, T. & Drakeley, C., 2011. Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination. *Clinical Microbiology Reviews*, 24(2), pp.377–410.
- Brannigan, J.A. et al., 2014. Diverse modes of binding in structures of *Leishmania* major N-myristoyltransferase with selective inhibitors. *IUCrJ*, 1(Pt 4), pp.250–260.
- Brasseur, P. et al., 1986. High Level of Sensitivity to Chloroquine of 72 *Plasmodium Falciparum* Isolates from Southern Cameroon in January 1985. *The American journal of tropical medicine and hygiene*, 35(4), pp.711–716.
- Broncel, M. et al., 2016. Global Profiling of Huntingtin-associated protein E (HYPE)-Mediated AMPylation through a Chemical Proteomic Approach. *Molecular & Cellular proteomics*, 15(2), pp.715–725.
- Broncel, M. et al., 2015. Multifunctional Reagents for Quantitative Proteome-Wide Analysis of Protein Modification in Human Cells and Dynamic Profiling of Protein Lipidation During Vertebrate Development. *Angewandte Chemie International Edition*, 54(20), pp.5948–5951.
- Brown, A.W., 1958. The insecticide-resistance problem: a review of developments in 1956 and 1957. *Bulletin of the World Health Organization*, 18(3), pp.309–321.
- Bryan, C.M. et al., 2011. High-throughput protein production and purification at the Seattle Structural Genomics Center for Infectious Disease. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, 67(Pt 9), pp.1010–1014.
- Bullen, H.E. et al., 2016. Phosphatidic Acid-Mediated Signaling Regulates Microneme Secretion in *Toxoplasma*. *Cell Host and Microbe*, 19(3), pp.349–360.
- Bunnag, D. et al., 1996. Quinine-tetracycline for multidrug resistant *falciparum* malaria. *The Southeast Asian journal of tropical medicine and public health*, 27(1), pp.15–18.

- Burghaus, P.A. & Lingelbach, K., 2001. Luciferase, When Fused to an N-terminal Signal Peptide, Is Secreted from Transfected *Plasmodium falciparum* and Transported to the Cytosol of Infected Erythrocytes. *Journal of Biological Chemistry*, 276(29), pp.26838–26845.
- Bushell, E., Gomes, A. R., Sanderson, T., Anar, B., Girling, G., Herd, C., et al. 2017. Functional Profiling of a *Plasmodium* Genome Reveals an Abundance of Essential Genes. *Cell*. 170(2), pp.260–272.e8.
- Cabrera, A. et al., 2012. Dissection of minimal sequence requirements for rhoptry membrane targeting in the malaria parasite. *Traffic*, 13(10), pp.1335–1350.
- Cai, H., Reinisch, K. & Ferro-Novick, S., 2007. Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Developmental cell*, 12(5), pp.671–682.
- Callebaut, I. et al., 2005. Prediction of the general transcription factors associated with RNA polymerase II in *Plasmodium falciparum*: conserved features and differences relative to other eukaryotes. *BMC genomics*, 6(1), p.100.
- Caminade, C. et al., 2014. Impact of climate change on global malaria distribution. *Proceedings of the National Academy of Sciences*, 111(9), pp.3286–3291.
- Cantin, G.T. & Yates, J.R., 2004. Strategies for shotgun identification of post-translational modifications by mass spectrometry. *Journal of chromatography. A*, 1053(1-2), pp.7–14.
- Cañas, B. et al., 2006. Mass spectrometry technologies for proteomics. *Briefings in functional genomics & proteomics*, 4(4), pp.295–320.
- Carter, R. & Mendis, K.N., 2002. Evolutionary and Historical Aspects of the Burden of Malaria. *Clinical Microbiology Reviews*, 15(4), pp.564–594.
- Casares, S., Brumeanu, T.-D. & Richie, T.L., 2010. The RTS,S malaria vaccine. *Vaccine*, 28(31), pp.4880–4894.
- Charman, S.A. et al., 2011. Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. *Proceedings of the National Academy of Sciences of the United States of America*, 108(11), pp.4400–4405.
- Chen, L. et al., 2011. An EGF-like Protein Forms a Complex with PfRh5 and Is Required for Invasion of Human Erythrocytes by *Plasmodium falciparum* M. J. Blackman, ed. *PLoS pathogens*, 7(9), p.e1002199.
- Chen, L., Qu, F.Y. & Zhou, Y.C., 1982. Field observations on the antimalarial piperazine. *Chinese medical journal*, 95(4), pp.281–286.
- Chen, V.B. et al., 2010. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta crystallographica. Section D, Biological crystallography*, 66(Pt 1), pp.12–21.
- Choi, R. et al., 2011. Immobilized metal-affinity chromatography protein-recovery screening is predictive of crystallographic structure success. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, 67(Pt 9), pp.998–1005.
- Ciechanover, A. & Ben-Saadon, R., 2004. N-terminal ubiquitination: more protein substrates join in. *Trends in cell biology*, 14(3), pp.103–106.
- Collins, C.R. et al., 2017. The *Plasmodium falciparum* pseudoprotease SERA5 regulates the kinetics and efficiency of malaria parasite egress from host erythrocytes. O. Billker, ed. *PLoS pathogens*, 13(7), p.e1006453.
- Collins, C.R., Das, S., et al., 2013. Robust inducible Cre recombinase activity in the human malaria parasite *Plasmodium falciparum* enables efficient gene deletion

- within a single asexual erythrocytic growth cycle. *Molecular Microbiology*, 88(4), pp.687–701.
- Collins, C.R., Hackett, F., et al., 2013. Malaria parasite cGMP-dependent protein kinase regulates blood stage merozoite secretory organelle discharge and egress. L. D. Sibley, ed. *PLoS pathogens*, 9(5), p.e1003344.
- Coteron, J.M. et al., 2011. Structure-Guided Lead Optimization of Triazolopyrimidine-Ring Substituents Identifies Potent Plasmodium falciparum Dihydroorotate Dehydrogenase Inhibitors with Clinical Candidate Potential. *Journal of Medicinal Chemistry*, 54(15), pp.5540–5561.
- Cowman, A.F. et al., 1991. A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. *The Journal of cell biology*, 113(5), pp.1033–1042.
- Cowman, A. F., Healer, J., Marapana, D., & Marsh, K. Malaria: Biology and Disease. *Cell*. (2016), 167(3), 610–624.
- Cowman, A.F. et al., 2017. The Molecular Basis of Erythrocyte Invasion by Malaria Parasites. *Cell Host and Microbe*, 22(2), pp.232–245.
- Cowman, A.F., Galatis, D. & Thompson, J.K., 1994. Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. *Proceedings of the National Academy of Sciences*, 91(3), pp.1143–1147.
- Crabb, B.S. et al., 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell*, 89(2), pp.287–296.
- Culleton, R. & Carter, R., 2012. African Plasmodium vivax: Distribution and origins. *International journal for parasitology*, 42(12), pp.1091–1097.
- Das, S. et al., 2015. Processing of Plasmodium falciparum Merozoite Surface Protein MSP1 Activates a Spectrin-Binding Function Enabling Parasite Egress from RBCs. *Cell Host and Microbe*, 18(4), pp.433–444.
- Davda, D. et al., 2013. Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate. *ACS Chemical Biology*, 8(9), pp.1912–1917.
- Deitsch, K., Driskill, C. & Wellems, T., 2001. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Research*, 29(3), pp.850–853.
- Diana Murray et al., 1998. Electrostatics and the Membrane Association of Src: Theory and Experiment†. *Biochemistry*, 37(8), pp.2145–2159.
- Doderer-Lang, C. et al., 2014. The ears of the African elephant: unexpected high seroprevalence of Plasmodium ovale and Plasmodium malariae in healthy populations in Western Africa. *Malaria Journal*, 13(1), p.240.
- Doench, J.G. et al., 2014. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation., 32(12), pp.1262–1267.
- Donaldson, J.G. & Jackson, C.L., 2011. ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nature Reviews Molecular cell biology*, 12(6), pp.362–375.
- Dondorp, A.M. et al., 2009. Artemisinin Resistance in Plasmodium falciparum Malaria. *New England Journal of Medicine*, 361(5), pp.455–467.
- Douglas, N.M. et al., 2010. Artemisinin combination therapy for vivax malaria. *The Lancet. Infectious diseases*, 10(6), pp.405–416.
- Drewry, L.L. & Sibley, L.D., 2015. Toxoplasma Actin Is Required for Efficient Host Cell Invasion. *mBio*, 6(3), p.e00557.

- Dvorak, J.A. et al., 1975. Invasion of erythrocytes by malaria merozoites. *Science*, 187(4178), pp.748–750.
- Dvorin, J.D. et al., 2010. A Plant-Like Kinase in *Plasmodium falciparum* Regulates Parasite Egress from Erythrocytes. *Science*, 328(5980), pp.910–912.
- Ebine, K. et al., 2016. *Plasmodium* Rab5b is secreted to the cytoplasmic face of the tubovesicular network in infected red blood cells together with N-acylated adenylate kinase 2. *Malaria Journal*, 15(1), p.323.
- Egan, T.J. et al., 2002. Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *Biochemical Journal*, 365(2), pp.343–347.
- Egarter, S. et al., 2014. The toxoplasma Acto-MyoA motor complex is important but not essential for gliding motility and host cell invasion. F. Frischknecht, ed. *PloS One*, 9(3), p.e91819.
- Emsley, P. & Cowtan, K., 2004. Coot: model-building tools for molecular graphics. *Acta crystallographica. Section D, Biological crystallography*, 60(Pt 12 Pt 1), pp.2126–2132.
- Escalante, A.A. et al., 2005. A monkey's tale: The origin of *Plasmodium vivax* as a human malaria parasite. *Proceedings of the National Academy of Sciences*, 102(6), pp.1980–1985.
- Escalante, A.A., Barrio, E. & Ayala, F.J., 1995. Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. *Molecular biology and evolution*, 12(4), pp.616–626.
- Eskenazi, B. et al., 2009. The Pine River Statement: Human Health Consequences of DDT Use. *Environmental Health Perspectives*, 117(9), pp.1359–1367.
- Evans, P., 2006. Scaling and assessment of data quality. *Acta crystallographica. Section D, Biological crystallography*, 62(Pt 1), pp.72–82.
- Eyles, D.E. et al., 1963. *Plasmodium Falciparum* Resistant to Chloroquine in Cambodia. *The American journal of tropical medicine and hygiene*, 12(6), pp.840–843.
- Ezougou, C.N. et al., 2014. *Plasmodium falciparum* Rab5B is an N-terminally myristoylated Rab GTPase that is targeted to the parasite's plasma and food vacuole membranes T. Tsuboi, ed. *PloS One*, 9(2), p.e87695.
- Fang, H. et al., 2017. Multiple short windows of Calcium-Dependent Protein Kinase 4 activity coordinate distinct cell cycle events during *Plasmodium* gametogenesis. *eLife*, 6, p.e26524.
- Feachem, R.G.A. et al., 2010. Call to action: priorities for malaria elimination. *Lancet (London, England)*, 376(9752), pp.1517–1521.
- Fidock, D.A. & Wellems, T.E., 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proceedings of the National Academy of Sciences*, 94(20), pp.10931–10936.
- Fidock, D.A. et al., 2000. Mutations in the *P. falciparum* Digestive Vacuole Transmembrane Protein PfCRT and Evidence for Their Role in Chloroquine Resistance. *Molecular cell*, 6(4), pp.861–871.
- Foley, M. & Tilley, L., 1997. Quinoline antimalarials: Mechanisms of action and resistance. *International journal for parasitology*, 27(2), pp.231–240.
- Frearson, J.A. et al., 2010. N-myristoyltransferase inhibitors as new leads to treat sleeping sickness. *Nature*, 464(7289), pp.728–732.
- Frénal, K. et al., 2010. Functional dissection of the apicomplexan glideosome molecular architecture. *Cell Host and Microbe*, 8(4), pp.343–357.

- Frénal, K. et al., 2017. Gliding motility powers invasion and egress in Apicomplexa. *Nature Reviews Microbiology*, 45, p.597.
- Frénal, K. et al., 2013. Global analysis of apicomplexan protein S-acyl transferases reveals an enzyme essential for invasion. *Traffic*, 14(8), pp.895–911.
- Galaway, F. et al., 2017. P113 is a merozoite surface protein that binds the N terminus of Plasmodium falciparum RH5. *Nature communications*, 8, p.14333.
- Gamo, F.-J. et al., 2010. Thousands of chemical starting points for antimalarial lead identification. *Nature*, 465(7296), pp.305–310.
- Gaskins, E. et al., 2004. Identification of the membrane receptor of a class XIV myosin in Toxoplasma gondii. *The Journal of cell biology*, 165(3), pp.383–393.
- Gentile, J.E., Rund, S.S.C. & Madey, G.R., 2015. Modelling sterile insect technique to control the population of Anopheles gambiae. *Malaria Journal*, 14(1), p.92.
- Ghorbal, M. et al., 2014. Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system., 32(8), pp.819–821.
- Ghosh, A.K., Ribolla, P.E.M. & Jacobs-Lorena, M., 2001. Targeting Plasmodium ligands on mosquito salivary glands and midgut with a phage display peptide library. *Proceedings of the National Academy of Sciences*, 98(23), pp.13278–13281.
- Giang, D.K. & Cravatt, B.F., 1998. A second mammalian N-myristoyltransferase. *Journal of Biological Chemistry*, 273(12), pp.6595–6598.
- Giglione, C., Fieulaine, S. & Meinnel, T., 2015. N-terminal protein modifications: Bringing back into play the ribosome. *Bulletin de la Société de Chimie Biologique*, 114, pp.134–146.
- Gisselberg, J.E. et al., 2017. The Prenylated Proteome of Plasmodium falciparum Reveals Pathogen-specific Prenylation Activity and Drug Mechanism-of-action. *Molecular & Cellular proteomics*, 16(4 suppl 1), pp.S54–S64.
- Glover, C.J., Hartman, K.D. & Felsted, R.L., 1997. Human N-myristoyltransferase amino-terminal domain involved in targeting the enzyme to the ribosomal subcellular fraction. *Journal of Biological Chemistry*, 272(45), pp.28680–28689.
- Gomes, A.R. et al., 2015. A genome-scale vector resource enables high-throughput reverse genetic screening in a malaria parasite. *Cell Host and Microbe*, 17(3), pp.404–413.
- Gomez, C. et al., 2010. Regulation of gene expression in protozoa parasites. *Journal of biomedicine & biotechnology*, 2010(12), pp.726045–24.
- Goncalves, V. et al., 2017. Structure-guided optimization of quinoline inhibitors of Plasmodium N -myristoyltransferase. *MedChemComm*, 8(1), pp.191–197.
- Goncalves, V., Brannigan, J.A., Thinon, E., et al., 2012. A fluorescence-based assay for N-myristoyltransferase activity. *Analytical biochemistry*, 421(1), pp.342–344.
- Goncalves, V., Brannigan, J.A., Whalley, D., et al., 2012. Discovery of Plasmodium vivaxN-Myristoyltransferase Inhibitors: Screening, Synthesis, and Structural Characterization of their Binding Mode. *Journal of Medicinal Chemistry*, 55(7), pp.3578–3582.
- Goya Grocin, A. et al., 2019. Whole Proteome Profiling of N-Myristoyltransferase Activity and Inhibition Using Sortase A. *Molecular & Cellular proteomics*, 18(1), pp.115–126.
- Green, J.L. et al., 2017. Compositional and expression analyses of the glideosome during the Plasmodium life cycle reveal an additional myosin light chain required for maximum motility. *Journal of Biological Chemistry*, 292(43), pp.17857–17875.

- Green, J.L. et al., 2015. Imidazopyridazine inhibitors of *Plasmodium falciparum* calcium dependent protein kinase 1 also target cGMP-dependent protein kinase and heat shock protein 90 to kill the parasite at different stages of intracellular development. *Antimicrobial Agents and Chemotherapy*, pp.AAC.01748–15.
- Green, J.L. et al., 2008. The motor complex of *Plasmodium falciparum*: phosphorylation by a calcium-dependent protein kinase. *Journal of Biological Chemistry*, 283(45), pp.30980–30989.
- Green, J.L. et al., 2006. The MTIP-myosin A complex in blood stage malaria parasites. *Journal of molecular biology*, 355(5), pp.933–941.
- Guiguemde, W.A. et al., 2010. Chemical genetics of *Plasmodium falciparum*. *Nature*, 465(7296), pp.311–315.
- Gunaratne, R.S. et al., 2000. Characterization of N-myristoyltransferase from *Plasmodium falciparum*. *Biochemical Journal*, 348 Pt 2(Pt 2), pp.459–463.
- Gupta, V.A. & Beggs, A.H., 2014. Kelch proteins: emerging roles in skeletal muscle development and diseases. *Skeletal muscle*, 4(1), p.11.
- Guttery, D.S. et al., 2014. Genome-wide functional analysis of *Plasmodium* protein phosphatases reveals key regulators of parasite development and differentiation. *Cell Host and Microbe*, 16(1), pp.128–140.
- Haldane, J.B.S., 1949. THE RATE OF MUTATION OF HUMAN GENES. *Hereditas*, 35(S1), pp.267–273.
- Haste, N.M. et al., 2012. Exploring the *Plasmodium falciparum* cyclic-adenosine monophosphate (cAMP)-dependent protein kinase (PfPKA) as a therapeutic target. *Microbes and infection*, 14(10), pp.838–850.
- Heal, W.P. et al., 2011. Bioorthogonal chemical tagging of protein cholesterylization in living cells. *Chemical Communications*, 47(14), pp.4081–4083.
- Heal, W.P. et al., 2008. N-Myristoyl transferase-mediated protein labelling in vivo. *Organic & biomolecular chemistry*, 6(13), pp.2308–2315.
- Heinberg, A. et al., 2013. Direct evidence for the adaptive role of copy number variation on antifolate susceptibility in *Plasmodium falciparum*. *Molecular Microbiology*, 88(4), pp.702–712.
- Herrera, L.J. et al., 2016. Validation of N-myristoyltransferase as Potential Chemotherapeutic Target in Mammal-Dwelling Stages of *Trypanosoma cruzi*. C. Franco-Paredes, ed. *PLoS neglected tropical diseases*, 10(4), p.e0004540.
- Heuckeroth, R.O. et al., 1988. 11-(Ethylthio)undecanoic acid. A myristic acid analogue of altered hydrophobicity which is functional for peptide N-myristoylation with wheat germ and yeast acyltransferase. *Journal of Biological Chemistry*, 263(5), pp.2127–2133.
- Hill, A.V. et al., 1991. Common west African HLA antigens are associated with protection from severe malaria. *Nature*, 352(6336), pp.595–600.
- Hoess, R.H., Wierzbicki, A. & Abremski, K., 1986. The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Research*, 14(5), pp.2287–2300.
- Holder, A., Mohd Ridzuan, M.A. & Green, J.L., 2012. Calcium dependent protein kinase 1 and calcium fluxes in the malaria parasite. *Microbes and infection*, 14(10), pp.825–830.
- Holder, A.A. & Freeman, R.R., 1982. Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody. *The Journal of experimental medicine*, 156(5), pp.1528–1538.

- Homewood, C.A. et al., 1972. Lysosomes, pH and the Anti-malarial Action of Chloroquine. *Nature*, 235(5332), pp.50–52.
- Howe, R. et al., 2013. Isoprenoid biosynthesis inhibition disrupts Rab5 localization and food vacuolar integrity in *Plasmodium falciparum*. *Eukaryotic Cell*, 12(2), pp.215–223.
- Ingram, V.M., 1959. Abnormal human haemoglobins. III the chemical difference between normal and sickle cell haemoglobins. *Biochimica et Biophysica Acta*, 36(2), pp.402–411.
- Jebiwott, S. et al., 2013. Plasmodium berghei calcium dependent protein kinase 1 is not required for host cell invasion. *PloS One*, 8(11), p.e79171.
- Johnson, D.R., Bhatnagar, R.S., et al., 1994. Genetic and Biochemical Studies of Protein N-Myristoylation. *Annual review of biochemistry*, 63(1), pp.869–914.
- Johnson, D.R., Knoll, L.J., et al., 1994. Genetic analysis of the role of *Saccharomyces cerevisiae* acyl-CoA synthetase genes in regulating protein N-myristoylation. *Journal of Biological Chemistry*, 269(27), pp.18037–18046.
- Jones, M.L. et al., 2012. Analysis of protein palmitoylation reveals a pervasive role in *Plasmodium development* and pathogenesis. *Cell Host and Microbe*, 12(2), pp.246–258.
- Jullien, N. et al., 2007. Conditional transgenesis using Dimerizable Cre (DiCre). P. Callaerts, ed. *PloS One*, 2(12), p.e1355.
- Jullien, N. et al., 2003. Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. *Protein engineering design and selection*, 31(21), p.e131.
- Kabsch, W., 2010. Integration, scaling, space-group assignment and post-refinement. *Acta crystallographica. Section D, Biological crystallography*, 66(2), pp.133–144.
- Kahn, R.A. & Gilman, A.G., 1984. Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *Journal of Biological Chemistry*, 259(10), pp.6228–6234.
- Kensche, P.R. et al., 2016. The nucleosome landscape of *Plasmodium falciparum* reveals chromatin architecture and dynamics of regulatory sequences. *Nucleic Acids Research*, 44(5), pp.2110–2124.
- Kishore, N.S. et al., 1993. Comparison of the acyl chain specificities of human myristoyl-CoA synthetase and human myristoyl-CoA:protein N-myristoyltransferase. *Journal of Biological Chemistry*, 268(7), pp.4889–4902.
- Klonis, N., Creek, D.J. & Tilley, L., 2013. Iron and heme metabolism in *Plasmodium falciparum* and the mechanism of action of artemisinins. *Current Opinion in Microbiology*, 16(6), pp.722–727.
- Knuepfer, E. et al., 2019. Divergent roles for the RH5 complex components, CyRPA and RIPR in human-infective malaria parasites. D. Soldati-Favre, ed. *PLoS pathogens*, 15(6), p.e1007809.
- Knuepfer, E. et al., 2017. Generating conditional gene knockouts in Plasmodium - a toolkit to produce stable DiCre recombinase-expressing parasite lines using CRISPR/Cas9. *Scientific reports*, 7(1), p.3881.
- Kuglstadt, D. et al., 2007. Raf kinase inhibitor protein affects activity of *Plasmodium falciparum* calcium-dependent protein kinase 1. *Molecular and Biochemical Parasitology*, 151(1), pp.111–117.
- Labuhn, M. et al., 2018. Refined sgRNA efficacy prediction improves large- and small-scale CRISPR-Cas9 applications. *Nucleic Acids Research*, 46(3), pp.1375–1385.

- Lagacé, L. et al., 2011. In Vitro Resistance Profile of the Hepatitis C Virus NS3 Protease Inhibitor BI 201335. *Antimicrobial Agents and Chemotherapy*, 56(1), pp.569–572.
- Lang, K. & Chin, J.W., 2014. Bioorthogonal reactions for labeling proteins., 9(1), pp.16–20.
- Lanyon-Hogg, T. et al., 2017. Dynamic Protein Acylation: New Substrates, Mechanisms, and Drug Targets. *Trends in biochemical sciences*, 42(7), pp.566–581.
- Lasonder, E. et al., 2012. Insights into the *Plasmodium falciparum* schizont phospho-proteome. *Microbes and infection*, 14(10), pp.811–819.
- Le Manach, C. et al., 2016. Identification of a Potential Antimalarial Drug Candidate from a Series of 2-Aminopyrazines by Optimization of Aqueous Solubility and Potency across the Parasite Life Cycle. *Journal of Medicinal Chemistry*, 59(21), pp.9890–9905.
- Leang, R. et al., 2013. Efficacy of Dihydroartemisinin-Piperaquine for Treatment of Uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010. *Antimicrobial Agents and Chemotherapy*, 57(2), pp.818–826.
- Lee, A.H., Symington, L.S. & Fidock, D.A., 2014. DNA repair mechanisms and their biological roles in the malaria parasite *Plasmodium falciparum*. *Microbiology and molecular biology reviews : MMBR*, 78(3), pp.469–486.
- Li, H. et al., 2016. Structure and function based design of *Plasmodium*-selective proteasome inhibitors. *Nature*, 530(7589), pp.233–236.
- Li, H. et al., 2012. Validation of the Proteasome as a Therapeutic Target in *Plasmodium* Using an Epoxyketone Inhibitor with Parasite-Specific Toxicity. *Chemistry & Biology*, 19(12), pp.1535–1545.
- Li, Q. et al., 2014. Assessment of the prophylactic activity and pharmacokinetic profile of oral tafenoquine compared to primaquine for inhibition of liver stage malaria infections. *Malaria Journal*, 13(1), p.141.
- Lim, M.Y.-X. et al., 2016. UDP-galactose and acetyl-CoA transporters as *Plasmodium* multidrug resistance genes. *Nature Microbiology*, 1(12), p.16166.
- Liu, W. et al., 2014. African origin of the malaria parasite *Plasmodium vivax*. *Nature communications*, 5(1), p.3346.
- Liu, W. et al., 2010. Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. *Nature*, 467(7314), pp.420–425.
- Loeb, F. et al., 1946. Activity of a New Antimalarial Agent, Chloroquine. (SN 7618): Statement Approved by the Board for Coordination of Malarial Studies. *Journal of the American Medical Association*, 130(16), pp.1069–1070.
- López, C. et al., 2010. Mechanisms of genetically-based resistance to malaria. *Gene*, 467(1-2), pp.1–12.
- Lumb, V. et al., 2011. Multiple Origins of *Plasmodium falciparum* Dihydropteroate Synthetase Mutant Alleles Associated with Sulfadoxine Resistance in India. *Antimicrobial Agents and Chemotherapy*, 55(6), pp.2813–2817.
- Ma, J. et al., 2012. Subcellular localization of adenylate kinases in *Plasmodium falciparum*. *FEBS Letters*, 586(19), pp.3037–3043.
- Macpherson, C.R. & Scherf, A., 2015. Flexible guide-RNA design for CRISPR applications using Protospacer Workbench., 33(8), pp.805–806.
- Marti, M. et al., 2005. Signal-mediated export of proteins from the malaria parasite to the host erythrocyte. *The Journal of cell biology*, 171(4), pp.587–592.

- Maurer-Stroh, S., Eisenhaber, B. & Eisenhaber, F., 2002a. N-terminal N-myristoylation of proteins: prediction of substrate proteins from amino acid sequence. *Journal of molecular biology*, 317(4), pp.541–557.
- Maurer-Stroh, S., Eisenhaber, B. & Eisenhaber, F., 2002b. N-terminal N-myristoylation of proteins: refinement of the sequence motif and its taxon-specific differences. *Journal of molecular biology*, 317(4), pp.523–540.
- Maxmen, A., 2019. First proven malaria vaccine rolled out in Africa - but doubts linger. *Nature*, 569(7754), pp.14–15.
- Mayxay, M. et al., 2004. Mixed-species malaria infections in humans. *Trends in parasitology*, 20(5), pp.233–240.
- Mbengue, A. et al., 2015. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. *Nature*, 520(7549), pp.683–687.
- McAlister, G.C. et al., 2014. MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Analytical chemistry*, 86(14), pp.7150–7158.
- McFadden, G.I. et al., 1996. Plastid in human parasites. *Nature*, 381(6582), pp.482–482.
- McNicholas, S. et al., 2011. Presenting your structures: the CCP4mg molecular-graphics software. *Acta crystallographica. Section D, Biological crystallography*, 67(Pt 4), pp.386–394.
- Medica, D.L. & Sinnis, P., 2005. Quantitative Dynamics of *Plasmodium yoelii* Sporozoite Transmission by Infected Anopheline Mosquitoes. *Infection and immunity*, 73(7), pp.4363–4369.
- Meshnick, S.R., 2002. Artemisinin: mechanisms of action, resistance and toxicity. *International journal for parasitology*, 32(13), pp.1655–1660.
- Méthot, N. et al., 2008. In Vivo Inhibition of Serine Protease Processing Requires a High Fractional Inhibition of Cathepsin C. *Molecular Pharmacology*, 73(6), pp.1857–1865.
- Méthot, N. et al., 2007. Inhibition of the activation of multiple serine proteases with a cathepsin C inhibitor requires sustained exposure to prevent pro-enzyme processing. *Journal of Biological Chemistry*, 282(29), pp.20836–20846.
- Miao, J. et al., 2006. The malaria parasite *Plasmodium falciparum* histones: Organization, expression, and acetylation. *Gene*, 369, pp.53–65.
- Michel, G. et al., 2010. Site-specific gene insertion mediated by a Cre-loxP-carrying lentiviral vector. *Molecular therapy : the journal of the American Society of Gene Therapy*, 18(10), pp.1814–1821.
- Miller, K.D., Greenberg, A.E. & Campbell, C.C., 1989. Treatment of Severe Malaria in the United States with a Continuous Infusion of Quinidine Gluconate and Exchange Transfusion. *New England Journal of Medicine*, 321(2), pp.65–70.
- Miller, L.H., Ackerman, H.C., Su, X.-Z. & Wellems, T.E., 2013a. Malaria biology and disease pathogenesis: insights for new treatments. *Nature medicine*, 19(2), pp.156–167.
- Miller, L.H., Ackerman, H.C., Su, X.Z. & Wellems, T.E., 2013b. Malaria biology and disease pathogenesis: insights for new treatments. *Nature medicine*, 19(2), pp.156–167.
- Moon, R.W. et al., 2013. Adaptation of the genetically tractable malaria pathogen *Plasmodium knowlesi* to continuous culture in human erythrocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 110(2), pp.531–536.

- Moore, R.B. et al., 2008. A photosynthetic alveolate closely related to apicomplexan parasites. *Nature*, 451(7181), pp.959–963.
- Mota, M.M. et al., 2001. Migration of Plasmodium Sporozoites Through Cells Before Infection. *Science*, 291(5501), pp.141–144.
- Motulsky, H.J. & Brown, R.E., 2006. Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. *BMC bioinformatics*, 7(1), p.123.
- Mousnier, A. et al., 2018. Fragment-derived inhibitors of human N-myristoyltransferase block capsid assembly and replication of the common cold virus. *Nature Chemistry*, 12(6), pp.1–606.
- Möskes, C. et al., 2004. Export of *Plasmodium falciparum* calcium-dependent protein kinase 1 to the parasitophorous vacuole is dependent on three N-terminal membrane anchor motifs. *Molecular microbiology*, 54(3), pp.676–691.
- Mueller, C. et al., 2016. Structural and functional dissection of *Toxoplasma gondii* armadillo repeats only protein. *Journal of cell science*, 129(5), pp.1031–1045.
- Mueller, C. et al., 2013. The Toxoplasma protein ARO mediates the apical positioning of rhoptry organelles, a prerequisite for host cell invasion. *Cell Host and Microbe*, 13(3), pp.289–301.
- Mueller, I., Zimmerman, P.A. & Reeder, J.C., 2007. *Plasmodium malariae* and *Plasmodium ovale* – the “bashful” malaria parasites. *Trends in parasitology*, 23(6), pp.278–283.
- Munhenga, G. et al., 2011. Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory colonized and a wild population of Anopheles arabiensis from the Kruger National Park, South Africa. *Parasites & vectors*, 4(1), p.208.
- Nair, S. et al., 2008. Adaptive copy number evolution in malaria parasites. M. Przeworski, ed. *PLoS genetics*, 4(10), p.e1000243.
- Nevin, R.L. & Croft, A.M., 2016. Psychiatric effects of malaria and anti-malarial drugs: historical and modern perspectives. *Malaria Journal*, 15(1), p.332.
- Ng, C.L., Fidock, D.A. & Bogyo, M., 2017. Protein Degradation Systems as Antimalarial Therapeutic Targets. *Trends in parasitology*, 33(9), pp.731–743.
- Noedl, H. et al., 2009. Evidence of Artemisinin-Resistant Malaria in Western Cambodia. *dx.doi.org*, 359(24), pp.2619–2620.
- Ntwasa, M. et al., 2001. Drosophila embryos lacking N-myristoyltransferase have multiple developmental defects. *Experimental cell research*, 262(2), pp.134–144.
- Ono, T. et al., 1993. Gametocytogenesis induction by Berenil in cultured *Plasmodium falciparum*. *Experimental Parasitology*, 77(1), pp.74–78.
- Paddon, C.J. & Keasling, J.D., 2014. Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. *Nature Reviews Microbiology*, 12(5), pp.355–367.
- Paul, P. et al., 2015. Homology modeling and molecular dynamics simulation of N-myristoyltransferase from Plasmodium falciparum: an insight into novel antimalarial drug design. *Journal of Molecular Modeling*, 21(3), p.37.
- Payne, D., 1987. Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitology today*, 3(8), pp.241–246.
- Pease, B.N. et al., 2013. Global Analysis of Protein Expression and Phosphorylation of Three Stages of Plasmodium falciparum Intraerythrocytic Development. *Journal of proteome research*, 12(9), pp.4028–4045.

- Pegoraro, S. et al., 2017. SC83288 is a clinical development candidate for the treatment of severe malaria. *Nature communications*, 8, p.14193.
- Peitzsch, R.M. & McLaughlin, S., 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry*, 32(39), pp.10436–10443.
- Percher, A. et al., 2016. Mass-tag labeling reveals site-specific and endogenous levels of protein S-fatty acylation. *Proceedings of the National Academy of Sciences of the United States of America*, 113(16), pp.4302–4307.
- Perrin, A.J. et al., 2018. The Actinomyosin Motor Drives Malaria Parasite Red Blood Cell Invasion but Not Egress. L. D. Sibley, ed. *mBio*, 9(4), p.47.
- Peterson, D.S., Walliker, D. & Wellems, T.E., 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proceedings of the National Academy of Sciences*, 85(23), pp.9114–9118.
- Phyo, A.P. et al., 2016. Antimalarial activity of artefenomel (OZ439), a novel synthetic antimalarial endoperoxide, in patients with *Plasmodium falciparum* and *Plasmodium vivax* malaria: an open-label phase 2 trial. *The Lancet. Infectious diseases*, 16(1), pp.61–69.
- Pino, P. et al., 2012. A Tetracycline-Repressible Transactivator System to Study Essential Genes in Malaria Parasites. *Cell Host and Microbe*, 12(6), pp.824–834.
- Ponnudurai, T. et al., 1991. Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85(2), pp.175–180.
- Poulin, B. et al., 2013. Unique apicomplexan IMC sub-compartment proteins are early markers for apical polarity in the malaria parasite. *Biology Open*, 2(11), pp.1160–1170.
- Price, H.P. et al., 2003. Myristoyl-CoA:protein N-myristoyltransferase, an essential enzyme and potential drug target in kinetoplastid parasites. *Journal of Biological Chemistry*, 278(9), pp.7206–7214.
- Qinghaosu Antimalaria Coordinating Research Group, 1979. Antimalaria studies on qinghaosu. *Chinese medical journal*, 92(12), pp.811–816.
- Quevillon, E. et al., 2003. The *Plasmodium falciparum* family of Rab GTPases. *Gene*, 306, pp.13–25.
- Rackham, M.D. et al., 2014. Design and synthesis of high affinity inhibitors of *Plasmodium falciparum* and *Plasmodium vivax* N-myristoyltransferases directed by ligand efficiency dependent lipophilicity (LELP). *Journal of Medicinal Chemistry*, 57(6), pp.2773–2788.
- Rackham, M.D. et al., 2013. Discovery of novel and ligand-efficient inhibitors of *Plasmodium falciparum* and *Plasmodium vivax* N-myristoyltransferase. *Journal of Medicinal Chemistry*, 56(1), pp.371–375.
- Rahlfs, S. et al., 2009. Myristoylated adenylate kinase-2 of *Plasmodium falciparum* forms a heterodimer with myristoyltransferase. *Molecular and Biochemical Parasitology*, 163(2), pp.77–84.
- Raibaud, A. et al., 2001. Cryofracture Electron Microscopy of the Ookinete Pellicle of *Plasmodium gallinaceum* Reveals the Existence of Novel Pores in the Alveolar Membranes. *Journal of structural biology*, 135(1), pp.47–57.
- Reddy, K.S. et al., 2015. Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium falciparum* erythrocyte

- invasion. *Proceedings of the National Academy of Sciences*, 112(4), pp.1179–1184.
- Rees-Channer, R.R. et al., 2006. Dual acylation of the 45 kDa gliding-associated protein (GAP45) in *Plasmodium falciparum* merozoites. *Molecular and Biochemical Parasitology*, 149(1), pp.113–116.
- Ren, J. et al., 2008. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein engineering design and selection*, 21(11), pp.639–644.
- Resh, M.D., 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochimica et Biophysica Acta*, 1451(1), pp.1–16.
- Resh, M.D., 1994. Myristylation and palmitylation of Src family members: The fats of the matter. *Cell*, 76(3), pp.411–413.
- Resh, M.D., 2006. Trafficking and signaling by fatty-acylated and prenylated proteins. *Nature chemical biology*, 2(11), pp.584–590.
- Rezvani, N. et al., 2012. Heterozygosity for the proteasomal Psmc1 ATPase is insufficient to cause neuropathology in mouse brain, but causes cell cycle defects in mouse embryonic fibroblasts. *Neuroscience letters*, 521(2), pp.130–135.
- Ridzuan, M.A.M. et al., 2012. Subcellular Location, Phosphorylation and Assembly into the Motor Complex of GAP45 during *Plasmodium falciparum* Schizont Development G. Langsley, ed. *PloS One*, 7(3), p.e33845.
- Roger, N. et al., 1988. Characterization of a 225 kilodalton rhoptry protein of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, 27(2-3), pp.135–141.
- Rosario, V., 1981. Cloning of naturally occurring mixed infections of malaria parasites. *Science*, 212(4498), pp.1037–1038.
- Rosenberg, R. & Rungsiwongse, J., 1991. The Number of Sporozoites Produced by Individual Malaria Oocysts. *The American journal of tropical medicine and hygiene*, 45(5), pp.574–577.
- Roth, A.F. et al., 2006. Global analysis of protein palmitoylation in yeast. *Cell*, 125(5), pp.1003–1013.
- Rudnick, D.A. et al., 1992. Analogs of palmitoyl-CoA that are substrates for myristoyl-CoA:protein N-myristoyltransferase. *Proceedings of the National Academy of Sciences of the United States of America*, 89(21), pp.10507–10511.
- Rudnick, D.A. et al., 1991. Kinetic and structural evidence for a sequential ordered Bi Bi mechanism of catalysis by *Saccharomyces cerevisiae* myristoyl-CoA:protein N-myristoyltransferase. *Journal of Biological Chemistry*, 266(15), pp.9732–9739.
- Russo, I., Oksman, A. & Goldberg, D.E., 2009. Fatty acid acylation regulates trafficking of the unusual *Plasmodium falciparum* calpain to the nucleolus. *Molecular Microbiology*, 72(1), pp.229–245.
- Russo, I., Oksman, A., Vaupel, B., et al., 2009. A calpain unique to alveolates is essential in *Plasmodium falciparum* and its knockdown reveals an involvement in pre-S-phase development. *Proceedings of the National Academy of Sciences of the United States of America*, 106(5), pp.1554–1559.
- Schindelin, J. et al., 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7), pp.676–682.
- Schlott, A., Tate, E.W. & Holder, A., 2015. *N-myristoyltransferase Inhibitor validation in the Malaria Parasite Plasmodium falciparum*,

- Schlott, A.C. et al., 2019. Structure-Guided Identification of Resistance Breaking Antimalarial *N*-Myristoyltransferase Inhibitors. *Cell chemical biology*.
- Schlott, A.C., Holder, A.A. & Tate, E.W., 2018. *N*-myristoylation as a drug target in malaria: exploring the role of *N*-myristoyltransferase substrates in inhibitor mode of action. *ACS infectious diseases*, 4(4), pp.449–457.
- Schwach, F. et al., 2015. PlasmoGEM, a database supporting a community resource for large-scale experimental genetics in malaria parasites. *Nucleic Acids Research*, 43, pp.D1176–82.
- Sebastian, S. et al., 2012. A *Plasmodium* Calcium-Dependent Protein Kinase Controls Zygote Development and Transmission by Translationally Activating Repressed mRNAs. *Cell Host and Microbe*, 12(1), pp.9–19.
- Seruggia, D. & Montoliu, L., 2014. The new CRISPR-Cas system: RNA-guided genome engineering to efficiently produce any desired genetic alteration in animals. *Transgenic research*, 23(5), pp.707–716.
- Sherling, E.S. et al., 2017. The *Plasmodium falciparum* rhoptry protein RhopH3 plays essential roles in host cell invasion and nutrient uptake. *eLife*, 6, p.D539.
- Shi, D.S. et al., 2014. Proteasome function is required for platelet production. *The Journal of clinical investigation*, 124(9), pp.3757–3766.
- Sidik, S.M. et al., 2016. A Genome-wide CRISPR Screen in *Toxoplasma* Identifies Essential Apicomplexan Genes. *Cell*, 166(6), pp.1423–1435.e12.
- Sidjanski, S. & Vanderberg, J.P., 1997. Delayed Migration of *Plasmodium* Sporozoites from the Mosquito Bite Site to the Blood. *The American journal of tropical medicine and hygiene*, 57(4), pp.426–429.
- Silmon de Monerri, N.C. et al., 2011. Global identification of multiple substrates for *Plasmodium falciparum* SUB1, an essential malarial processing protease. J. H. Adams, ed. *Infection and immunity*, 79(3), pp.1086–1097.
- Sinha, A., Hughes, K. R., Modrzynska, K. K., Otto, T. D., Pfander, C., Dickens, N. J., et al. 2014. A cascade of DNA-binding proteins for sexual commitment and development in *Plasmodium*. *Nature*, 507(7491), pp. 253–257.
- Slater, A.F.G., 1993. Chloroquine: Mechanism of drug action and resistance in *plasmodium falciparum*. *Pharmacology & Therapeutics*, 57(2-3), pp.203–235.
- Slater, A.F.G. & Cerami, A., 1992. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. *Nature*, 355(6356), pp.167–169.
- Smilkstein, M. et al., 2004. Simple and Inexpensive Fluorescence-Based Technique for High-Throughput Antimalarial Drug Screening. *Antimicrobial Agents and Chemotherapy*, 48(5), pp.1803–1806.
- Spangenberg, T. et al., 2013. The Open Access Malaria Box: A Drug Discovery Catalyst for Neglected Diseases B. Russell, ed. *PloS One*, 8(6), p.e62906.
- Starheim, K.K., Gevaert, K. & Arnesen, T., 2012. Protein N-terminal acetyltransferases: when the start matters. *Trends in biochemical sciences*, 37(4), pp.152–161.
- Sternberg, N. & Hamilton, D., 1981. Bacteriophage P1 site-specific recombination: I. Recombination between loxP sites. *Journal of molecular biology*, 150(4), pp.467–486.
- Sternglanz, R. & Schindelin, H., 1999. Structure and mechanism of action of the histone acetyltransferase Gcn5 and similarity to other N-acetyltransferases. *Proceedings of the National Academy of Sciences*, 96(16), pp.8807–8808.

- Struck, N.S. et al., 2008. *Plasmodium falciparum* possesses two GRASP proteins that are differentially targeted to the Golgi complex via a higher- and lower-eukaryote-like mechanism. *Journal of cell science*, 121(Pt 13), pp.2123–2129.
- Struck, N.S. et al., 2005. Re-defining the Golgi complex in *Plasmodium falciparum* using the novel Golgi marker PfGRASP. *Journal of cell science*, 118(Pt 23), pp.5603–5613.
- Sturm, A. et al., 2006. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science (New York, N.Y.)*, 313(5791), pp.1287–1290.
- Su, X.-Z. et al., 1995. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of plasmodium falciparum-infected erythrocytes. *Cell*, 82(1), pp.89–100.
- Sutherland, C.J. et al., 2010. Two nonrecombining sympatric forms of the human malaria parasite *Plasmodium ovale* occur globally. *The Journal of Infectious Diseases*, 201(10), pp.1544–1550.
- Swinney, D.C., 2013. Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clinical pharmacology and therapeutics*, 93(4), pp.299–301.
- Takamune, N. et al., 2010. Suppression of human immunodeficiency virus type-1 production by coexpression of catalytic-region-deleted N-myristoyltransferase mutants. *Biological & pharmaceutical bulletin*, 33(12), pp.2018–2023.
- Tamkun, J.W. et al., 1991. The arflike gene encodes an essential GTP-binding protein in Drosophila. *Proceedings of the National Academy of Sciences*, 88(8), pp.3120–3124.
- Tang, H. & Han, M., 2017. Fatty Acids Regulate Germline Sex Determination through ACS-4-Dependent Myristoylation. *Cell*, 169(3), pp.457–469.e13.
- Tate, E.W. et al., 2013. N-Myristoyltransferase as a potential drug target in malaria and leishmaniasis. *Parasitology*, 141(1), pp.37–49.
- Tavares, J. et al., 2013. Role of host cell traversal by the malaria sporozoite during liver infection. *The Journal of experimental medicine*, 210(5), pp.905–915.
- Thalia A Farazi, Jill K Manchester, A. & Gordon, J.I., 2000. *Transient-State Kinetic Analysis of Saccharomyces cerevisiae MyristoylCoA:Protein N-myristoyltransferase Reveals that a Step after Chemical Transformation Is Rate Limiting*, American Chemical Society.
- Tham, W.-H., Healer, J. & Cowman, A.F., 2012. Erythrocyte and reticulocyte binding-like proteins of *Plasmodium falciparum*. *Trends in parasitology*, 28(1), pp.23–30.
- Thavayogarah, T. et al., 2015. Alternative protein secretion in the Malaria Parasite *Plasmodium falciparum* É. M. Braga, ed. *PLoS One*, 10(4), p.e0125191.
- The RTS, S.C.T.P.2., 2014. Efficacy and Safety of the RTS,S/AS01 Malaria Vaccine during 18 Months after Vaccination: A Phase 3 Randomized, Controlled Trial in Children and Young Infants at 11 African Sites S. Krishna, ed. *PLoS medicine*, 11(7), p.e1001685.
- Thinon, E. et al., 2014. Global profiling of co- and post-translationally N-myristoylated proteomes in human cells. *Nature communications*, 5, p.4919.
- Thinon, E. et al., 2016. N-Myristoyltransferase Inhibition Induces ER-Stress, Cell Cycle Arrest, and Apoptosis in Cancer Cells. *ACS Chemical Biology*, 11(8), pp.2165–2176.
- Thinon, E. et al., 2018. Selective Enrichment and Direct Analysis of Protein S-Palmitoylation Sites. *Journal of proteome research*, 17(5), pp.1907–1922.

- Thomas, D.C. et al., 2012. Regulation of *Plasmodium falciparum* glideosome associated protein 45 (PfGAP45) phosphorylation. I. Blader, ed. *PloS One*, 7(4), p.e35855.
- Thomas, J.A. et al., 2018. A protease cascade regulates release of the human malaria parasite *Plasmodium falciparum* from host red blood cells. *Nature Microbiology*, 10, p.1925.
- Thomas, J.A. et al., 2016. Development and Application of a Simple Plaque Assay for the Human Malaria Parasite *Plasmodium falciparum* T. Spielmann, ed. *PloS One*, 11(6), p.e0157873.
- Thompson, A. et al., 2003. Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Analytical chemistry*, 75(8), pp.1895–1904.
- Tilley, L. et al., 2016. Artemisinin Action and Resistance in *Plasmodium falciparum*. *Trends in parasitology*, 32(9), pp.682–696.
- Ting, L. et al., 2011. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nature Methods*, 8(11), pp.937–940.
- Tomavo, S. et al., 2013. Protein trafficking through the endosomal system prepares intracellular parasites for a home invasion. C. E. Chitnis, ed. *PLoS pathogens*, 9(10), p.e1003629.
- Tonkin, M.L. et al., 2011. Host Cell Invasion by Apicomplexan Parasites: Insights from the Co-Structure of AMA1 with a RON2 Peptide. *Science*, 333(6041), pp.463–467.
- Towler, D.A. et al., 1987. Amino-terminal processing of proteins by *N*-myristoylation. Substrate specificity of *N*-myristoyltransferase. *Journal of Biological Chemistry*, 262(3), pp.1030–1036.
- Trager, W. & Jensen, J.B., 1976. Human malaria parasites in continuous culture. *Science*, 193(4254), pp.673–675.
- Treeck, M. et al., 2011. The Phosphoproteomes of *Plasmodium falciparum* and *Toxoplasma gondii* Reveal Unusual Adaptations Within and Beyond the Parasites' Boundaries. *Cell Host and Microbe*, 10(4), pp.410–419.
- Trenholme, C.M. et al., 1975. Mefloquine (WR 142,490) in the treatment of human malaria. *Science*, 190(4216), pp.792–794.
- Triglia, T. et al., 1997. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences*, 94(25), pp.13944–13949.
- Tschan, S. et al., 2013. Broad-Spectrum Antimalarial Activity of Peptido Sulfonyl Fluorides, a New Class of Proteasome Inhibitors. *Antimicrobial Agents and Chemotherapy*, 57(8), pp.3576–3584.
- Tse, E.G., Korsik, M. & Todd, M.H., 2019. The past, present and future of anti-malarial medicines. *Malaria Journal*, 18(1), p.93.
- Tuikue Ndam, N. et al., 2017. Parasites Causing Cerebral *Falciparum* Malaria Bind Multiple Endothelial Receptors and Express EPCR and ICAM-1-Binding PfEMP1. *The Journal of Infectious Diseases*, 215(12), pp.1918–1925.
- Ud-Din, A.I.M.S., Tikhomirova, A. & Roujeinikova, A., 2016. Structure and Functional Diversity of GCN5-Related N-Acetyltransferases (GNAT). *International Journal of Molecular Sciences*, 17(7), p.1018.
- Utsumi, T. et al., 2001. Amino acid residue penultimate to the amino-terminal gly residue strongly affects two cotranslational protein modifications, *N*-myristoylation and *N*-acetylation. *Journal of Biological Chemistry*, 276(13), pp.10505–10513.

- Vagin, A. & Lebedev, A., 2015. MoRDa, an automatic molecular replacement pipeline. *Acta Crystallographica Section A*, A71(s19).
- Vaidya, A.B., 2011. Naphthoquinones: Atovaquone, and Other Antimalarials Targeting Mitochondrial Functions. In *Treatment and Prevention of Malaria*. Basel: Springer, Basel, pp. 127–139.
- van Agthoven, M.A. et al., 2019. Two-dimensional mass spectrometry: new perspectives for tandem mass spectrometry. *European biophysics journal : EBJ*, 48(3), pp.213–229.
- van den Berg, H., 2009. Global Status of DDT and Its Alternatives for Use in Vector Control to Prevent Disease. *Environmental Health Perspectives*, 117(11), pp.1656–1663.
- Wang, J. et al., 2015. Haem-activated promiscuous targeting of artemisinin in *Plasmodium falciparum*. *Nature communications*, 6(1), p.10111.
- Weinberg, R.A. et al., 1995. Genetic studies reveal that myristoylCoA:protein N-myristoyltransferase is an essential enzyme in *Candida albicans*. *Molecular microbiology*, 16(2), pp.241–250.
- Weiss, G.E. et al., 2015. Revealing the Sequence and Resulting Cellular Morphology of Receptor-Ligand Interactions during *Plasmodium falciparum* Invasion of Erythrocytes M. J. Blackman, ed. *PLoS pathogens*, 11(2), p.e1004670.
- Weissman, T.A. & Pan, Y.A., 2015. Brainbow: new resources and emerging biological applications for multicolor genetic labeling and analysis. *Genetics*, 199(2), pp.293–306.
- Werling, K. et al., 2019. Steroid Hormone Function Controls Non-competitive *Plasmodium* Development in *Anopheles*. *Cell*, 177(2), pp.315–325.e14.
- Wetzel, J. et al., 2015. The role of palmitoylation for protein recruitment to the inner membrane complex of the malaria parasite. *Journal of Biological Chemistry*, 290(3), pp.1712–1728.
- White, N.J., 2018. Anaemia and malaria. *Malaria Journal*, 17(1), p.371.
- White, N.J. et al., 2014. Malaria. *Lancet (London, England)*, 383(9918), pp.723–735.
- Wickham, M.E., Culvenor, J.G. & Cowman, A.F., 2003. Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. *Journal of Biological Chemistry*, 278(39), pp.37658–37663.
- Wilcox, C., Hu, J.S. & Olson, E.N., 1987. Acylation of proteins with myristic acid occurs cotranslationally. *Science*, 238(4831), pp.1275–1278.
- Wong, T.W.T., 2015. *Essential Residues in IMC Recruitment of PfGAP45 in the Malaria Parasite*,
- Wong, W. et al., 2017. Mefloquine targets the *Plasmodium falciparum* 80S ribosome to inhibit protein synthesis. *Nature Microbiology*, 2(6), p.nmicrobiol201731.
- World Health Organization, 2018a. *High burden to high impact: a targeted malaria response*, Mozambique.
- World Health Organization, 2011. *World Malaria Report 2011*,
- World Health Organization, 2015. *World Malaria Report 2015*,
- World Health Organization, 2016. *World Malaria Report 2016*, Geneva, Switzerland.
- World Health Organization, 2017. *World Malaria Report 2017*, Geneva, Switzerland.

- World Health Organization, 2018b. *World malaria report 2018*, Geneva, Switzerland.
- Wright, M.H., 2013. *Chemical tools for probing protein N-myristoylation in protozoan parasites - PhD. Thesis*. Imperial College London.
- Wright, M.H. et al., 2015. Global Analysis of Protein N-Myristoylation and Exploration of N-Myristoyltransferase as a Drug Target in the Neglected Human Pathogen *Leishmania donovani*. *Chemistry & Biology*, 22(3), pp.342–354.
- Wright, M.H. et al., 2010. Protein myristoylation in health and disease., 3(1), pp.19–35.
- Wright, M.H. et al., 2014. Validation of N-myristoyltransferase as an antimalarial drug target using an integrated chemical biology approach. *Nature Chemistry*, 6(2), pp.112–121.
- Wu, Q. et al., 2012. Recent advances on multidimensional liquid chromatography-mass spectrometry for proteomics: from qualitative to quantitative analysis--a review. *Analytica chimica acta*, 731, pp.1–10.
- Wu, Y. et al., 2016. Resistance to Mutant Group 2 Influenza Virus Neuraminidases of an Oseltamivir-Zanamivir Hybrid Inhibitor. A. García-Sastre, ed. *Journal of virology*, 90(23), pp.10693–10700.
- Wurtz, N. et al., 2011. cAMP-dependent protein kinase from *Plasmodium falciparum*: an update. *Parasitology*, 138(1), pp.1–25.
- Wühr, M. et al., 2012. Accurate multiplexed proteomics at the MS2 level using the complement reporter ion cluster. *Analytical chemistry*, 84(21), pp.9214–9221.
- Yang, S.H. et al., 2005. N-myristoyltransferase 1 is essential in early mouse development. *Journal of Biological Chemistry*, 280(19), pp.18990–18995.
- Yates, J.R., 2019. Recent technical advances in proteomics. *F1000Research*, 8, p.351.
- Yeoh, S. et al., 2007. Subcellular Discharge of a Serine Protease Mediates Release of Invasive Malaria Parasites from Host Erythrocytes. *Cell*, 131(6), pp.1072–1083.
- Yeoman, J.A. et al., 2011. Tracking Glideosome-Associated Protein 50 Reveals the Development and Organization of the Inner Membrane Complex of *Plasmodium falciparum*. *Eukaryotic Cell*, 10(4), pp.556–564.
- Yu, Z. et al., 2012. Design and Synthesis of Inhibitors of *Plasmodium falciparum* N-myristoyltransferase, a Promising Target for Anti-Malarial Drug Discovery. *Journal of Medicinal Chemistry*, 55(20), pp.8879–8890.
- Yuthavong, Y. et al., 2012. Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proceedings of the National Academy of Sciences of the United States of America*, 109(42), pp.16823–16828.
- Zhang, M. et al., 2018. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science (New York, N.Y.)*, 360(6388), p.eaap7847.
- Zhu, G., Marchewka, M.J. & Keithly, J.S., 2000. *Cryptosporidium parvum* appears to lack a plastid genome. *Microbiology (Reading, England)*, 146 (Pt 2)(2), pp.315–321.