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Shedding light on *Plasmodium knowlesi* food vacuoles

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Author’s declaration

I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or materials than those expressly indicated. I have not applied to be examined at any other institution, nor have I used the dissertation in this or any other form at any other institution as an examination paper, nor submitted it to any other faculty as a dissertation.

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Summary

The food vacuole, the site of haemoglobin degradation in malaria parasites is unique to the *Plasmodium* genus and therefore represents an invaluable target for the development of antimalarial compounds. This organelle and the essential process which occur within it, have been well characterised in *P. falciparum*, however knowledge is lacking as to the nature of this cellular compartment in other human malaria species. Due to the restricted host cell preference of *P. vivax*, *in vitro* culture of the parasite remains a challenge. The *in vitro* culture-adapted strains of the closely related primate malaria *P. knowlesi* and its recently developed transfection system, identify this parasite as a versatile tool to study "vivax-like" parasite biology. In the studies described in this thesis, the food vacuole biology of *P. knowlesi* (which also causes zoonotic disease in humans) is investigated to highlight differences in this organelle which may exist between parasite species.

To compare the food vacuole morphology of the phylogenetically related parasites *P. vivax*, *P. knowlesi* and *P. cynomolgi* with *P. falciparum,* a combination of staining techniques and expression of fluorescent fusion food vacuole-resident proteins with live confocal microscopy is used. Multiple food vacuoles which are actively degrading haemoglobin were observed in the "vivax-type" parasites, in contrast to a single, large food vacuole in *P. falciparum*. Electron microscopy is also used to complement these studies.

PlasmepsinIV (PM4) is one of the key enzymes involved in haemoglobin degradation. The *pkpm4* locus in *P. knowlesi* was modified to generate a copy of *pkpm4* tagged with the FKBP (FK506 binding protein)-destabilisation domain (DD), creating parasites (pkpm4DD) which express an unstable PkPM4 protein which is only stabilised in the presence of the compound Shield-1. In amino acid-limited medium, parasites depend on haemoglobin degradation for a source of amino acids. In the absence of Shield-1, the parasites remain viable but there is a detrimental effect on both appearance and growth rate of pkpm4DD parasites in amino acid-limited medium, while the wild-type *P. knowlesi* remains unaffected, highlighting the role of PkPM4 in haemoglobin degradation, but suggesting that the enzyme is not essential for asexual viability.

The food vacuole is also the proposed target for chloroquine, a drug which was successfully used to treat human malaria for many years, but is now dramatically less efficient for the treatment of *P. falciparum* due to the emergence of resistance. Mutations in PfCRT, a channel-like protein on the food vacuole membrane are implicated in chloroquine resistance in *P. falciparum* although no mutations in the PvCRT homologue have been identified in resistant *P. vivax*, indicating a difference in resistance mechanism of both parasites. To evaluate if PfCRT is capable of modulating drug susceptibility in a "vivax-type" parasite, a chloroquine resistant PfCRT allele was overexpressed in *P. knowlesi*, resulting in a four-fold increase in tolerance to chloroquine, indicating that PfCRT is capable of modulating chloroquine sensitivity in a "non-falciparum" parasite.

These studies demonstrate the value of a versatile *in vitro* culture and transfection system in a parasite phyogenetically closely related to the second most important human malaria parasite, and utilise it to highlight some key differences which exist between malaria species in an important drug target. Research on *P. knowlesi* into fundamental biological processes can provide important insight into "vivax-like" parasite biology, as well as provide an indispensable tool for drug and vaccine studies.

Zusammenfassung

Die Nahrungsvakuole ist der Ort an dem der Abbau des Hämoglobins im Malaria Parasiten stattfindet; diese Vakuole ist einzigartig für den Genus Plasnodium und repräsentiert somit ein unschätzbares Ziel zur Entwicklung für Anti-Malaria Komponenten. Diese Organelle und der wichtige Prozess, der in ihr ablauft, ist sehr gut karakterisiert für *P. falciparum*, für andere Malaria Arten des Menschen jedoch fehlt das Wissen über die Art dieser zellulären Komponente. Auf Grund der Vorliebe von *P vivax* für eine bestimmte Wirtszelle, bleibt die *in vitro* Kultur des Parasiten eine Herausforderung. Ein Ausweg bietet der nah verwandte Malaria Parasit, *P. knowlesi:* Die an eine *in vitro* Kultur angepassten Stämme und ein vor kurzem entwickeltes Transfektionssystems kennzeichnen diesen Parasiten als vielseitiges Werkzeug, um eine 'vivax-ähnliche' Biologie zu studieren. Die Biologie der Nahrungsvakuole von *P. knowlesi,* der auch Zoonose-Krankheiten beim Menschen verursachen kann, wird in den Studien zu dieser Diplomarbeit beschrieben; die Zielsetzung der Arbeit war es, die Unterschiede hervorzuheben, die zwischen verschiedenen Parasitenarten bestehen können.
Um die Morphologie der Nahrungsvakuolen der phylogenetisch verwandten Parasiten *P. vivax*, *P. knowlesi* und *P. cynomolgi* mit *P. falciparum* zu vergleichen, ist eine Kombination von Färbetechnicken und 'life-time' konfokaler Mikroskopie verwendet worden, die die Expression von fluoreszierenden Fusionsproteinen innerhalb der Vakuole zeigt. Verschiedene Arten von Nahrungsvakuolen, die aktief Hämoglobin abbauen, waren in Parasiten vom 'vivax'-Typ zu beobachten, im Gegensatz zu einer einzigen, großen Nahrungsvakuole, die in *P. falciparum* zu sehen war. Zusätzlich ist Elektronenmikroskopie verwendet worden, um diese Studien zu ergänzen.
PlasmepsinIV (PM4) ist eines der Schlüsselenzyme, die am Abbau von Hämoglobin beteiligt sind. Das *pkpm4* Gen in *P. knowlesi* wurde so verändert, dass es eine Kopie von *pkpm4* hervorbringt, die eine FKBP ('FK506 binding protein')-Destabilisierungsdomäne (DD) enthält. Dieses veränderte Gen kreiert Parasiten, die ein unstabiles PkPM4 Protein hervorbringen, das nur in Gegenwart von der Komponente Shield-1 stabil ist. In einem aminosäurelimitierenden Medium sind Parasiten abhängig von dem Abbau des Hämoglobins als Aminosauerequelle. In Abwesenheit von Shield-1 bleiben die Parasiten lebensfähig, aber ein aminosäurelimitierendes-Medium wirkt sich negativ sowohl auf das Aussehen wie auf das Wachstum der pkpm4DD Parasiten aus, während der Wildtyp *P. knowlesi* unberührt bleibt. Hiermit wird die Rolle des PKPM4 für den Aminosäureabbau unterstrichen Dies Resultat suggeriert aber auch, daß das Enzym nicht essentiell ist für die asexuelle Lebensfähigkeit des Parasiten.
Die Nahrungsvakuole wird auch als Ziel für Chloroquine gesehen, ein Medikament, das viele Jahre lang erfolgreich eingesetzt wurde, um die menschliche Malaria zu behandeln. Durch das Entstehen von Resistenzen ist Chloroquine heutzutage weniger effizient in der Behandelung von Malaria verursacht durch *P. falciparum*. Mutationen in PfCRT, einem 'channel-like' Protein auf der Membran der Nahrungsvakuole sind in die Chloroquine-Resistenz bei *P. falciparum* verwickelt, obwohl keine Mutationen in dem homologen PvCRT Protein in resistenten *P. vivax* Parasiten identifiziert wurden, was auf einen Unterschied im Resistenz-Mechanismus der beiden Parasiten hinweist. Um zu bewerten, ob PfCRT fähig ist die Empfindlichkeit gegen das Medikament zu verändern, wurde ein Chloroquine-resistentes PfCRT Allel in *P. knowlesi* überexprimiert. Dieser Versuch resultierte in einer vierfach ansteigenden Toleranz gegen Chloroquine in einem Parasiten vom ‚vivax-Typ', was darauf

hinweist, dass PfCRT fähig ist, die Chloroquine Toleranz in einem ‚non-falciparum' Parasiten zu modifizieren.
Diese Studien zeigen die Bedeuting eines vielseitigen *in vitro* Kultur- und Transfektionssystems in einem Parasiten, der phylogenetisch nah verwandt ist mit dem zweitwichtigsten Malariaparasiten, und nutzen es um einige Hauptunterschiede hervorzuheben, die zwischen verschiedene Parasiten bestehen bezüglich der Zielorganelle für das Medikament. Forschung an fundamentalen biologischen Prozessen von *P. knowlesi* kann uns Einsichten verschaffen in die Biologie von ‚vivax-ähnlichen' Parasiten und uns zudem ein unverzichtbares Hilfsmittel zur Verfügung stellen für Studien an Medikamenten und Impfstoffen.

Abbreviations

°C	degrees Celsius
2D	two dimensional
3D	three dimensional
APAD	3-acetylpyridine adenine dinucleotide
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary deoxy-ribonucleic acid
CQR	chloroquine resistant
CQS	chloroquine sensitive
C-terminal	carboxy-terminal
D. discoideum	*Dictyostelium discoideum*
DD	destabilisation domain
DIC	differential interference contrast
DIG	digoxygenin
DNA	deoxy-ribonucleic acid
ef1α	elongation factor 1 alpha
EM	electron microscopy
ES	early schizont
FKBP	FK506 binding protein
FPIX	ferric-protoporphyrin IX
FRAP	fluorescence recovery after photobleaching
GFP	green fluorescent protein
h-dhfr	human dihydrofolate reductase
HDP	Heme detoxification protein
IC_{50}	half maximal inhibitory concentration
IFA	immunofluorescence assay
kb	kilobase
KO	knock-out
kV	kilovolt
l	litre
LS	late schizont
LS Blue	LysoSensor Blue
M	molar
MACS	Magnetic cell separation
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
NBF	nitro blue formazan
NBT	nitro blue tetrazolium
ng	nanogram
nM	nanomolar
OD	optical density
ORF	open reading frame
P. berghei	*Plasmodium berghei*
P. cynomolgi	*Plasmodium cynomolgi*
P. falciparum	*Plasmodium falciparum*
P. gallinaceum	*Plasmodium gallinaceum*
P. knowlesi	*Plasmodium knowlesi*
P. lophurae	*Plasmodium lophurae*

P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PBS	phosphate buffered saline
Pc	*Plasmodium cynomolgi*
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PfCRT	*P. falciparum* chloroquine resistance transporter
PfFP2	*P. falciparum* falcipain 2
PfFP2'	*P. falciparum* falcipain 2'
PfFP3	*P. falciparum* falcipain 3
PfHAP	*P. falciparum* histo-aspartic protease
PfHRPII	*P. falciparum* histidine-rich protein II
PfHRPIII	*P. falciparum* histidine-rich protein III
PfPM1	*P. falciparum* plasmepsin I
PfPM2	*P. falciparum* plasmepsin II
PfPM4	*P. falciparum* plasmepsin IV
Pk	*Plasmodium knowlesi*
PkCRT	*P. knowlesi* chloroquine resistance transporter
*p*LDH	*Plasmodium* lactate dehydrogenase
Pv	*Plasmodium vivax*
PvCRT-o	*P. vivax* chloroquine resistance transporter
R	ring
RPMI-1640	Roswell Park Memorial Institute culture medium
RPMI-1AA	Amino-acid limited Roswell Park Memorial Institute culture medium
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate buffer
T	trophozoite
tg-dhfr/ts	*Toxoplasma gondii* dihydrofolate reductase thymidine synthase
UTR	untranslated region
UV	ultraviolet
µF	micro Faraday
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre

Contents

1. Introduction

1.1 Malaria, a devastating human disease

Malaria, caused by the protozoan parasite *Plasmodium*, is a disease which has plagued humanity throughout human history. The very earliest evidence of a *Plasmodium* parasite was found in a fossilized *Culex* mosquito in a piece of amber approximately 30 million years old (131). It has maintained a strong selective force on human evolution, evident from the presence of conditions such as sickle-cell disease, thalassaemias and glucose-6-dehydrogenase deficiency, conditions which persist due to the small reduction in susceptibility to malaria which they confer (91). References to the characteristic periodic fevers associated with malaria exist in many ancient Greek, Roman, Assyrian, Indian and Arabic writings, including the earliest from 5,000 years ago in the Chinese Huangdi Neijing (35), and evidence of malaria in ancient Egypt has been found with the identification of *Plasmodium* DNA in ancient Egyptian mummies (116).

Today, 3.3 billion people live at risk of malaria infection. In 2008 approximately 243 million cases resulted in almost 1 million deaths, 85% of which were children under the age of five, living in Sub-Saharan Africa (191). Malaria is predominantly a disease of the developing world, and its presence perpetuates the cycle of poverty. Illness and mortality due to malaria directly costs the African nations 12 billion US dollars per year and impedes the economic growth which could help these countries to eradicate the disease (60).

These parasites have evolved clever strategies to evade the immune system, challenging vaccine development. The panel of antimalarial drugs is rapidly becoming less effective worldwide due to the appearance and dissemination of drug resistant strains. Therefore there is an urgent need to identify the unique aspects of the parasite biology in order to develop new drugs and an effective vaccine.

The four human *Plasmodium* species are *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. The primate malaria *P. knowlesi* is considered the "fifth human malaria" since it has recently emerged as the cause of a large number of malaria cases in South East Asia (182). *P. falciparum* is responsible for the most morbidity and accounts for 90% of the deaths attributed to malaria. *P. falciparum* is endemic in many tropical regions; throughout most of Sub-Saharan Africa, South and East Asia and South America (Fig. 1). *P. vivax* is the second most common cause of malaria and although it is less prevalent in Africa, it extends into many temperate areas in Asia and the Americas (Fig. 1). *Plasmodium* parasites have a complex life cycle with two hosts (Fig. 2). Transmitted to humans by the bite of a mosquito, the infection begins with an asymptomatic liver stage followed by the blood stages of the parasite which are responsible for the symptoms of the disease. Symptoms include the characteristic recurrent fevers, headaches, chills, joint pain, vomiting, anaemia, retinal damage and convulsions. The high mortality associated with *P. falciparum* infections is due to its ability to sequester in capillaries in major organs such as the brain, leading to damage, organ failure and death.

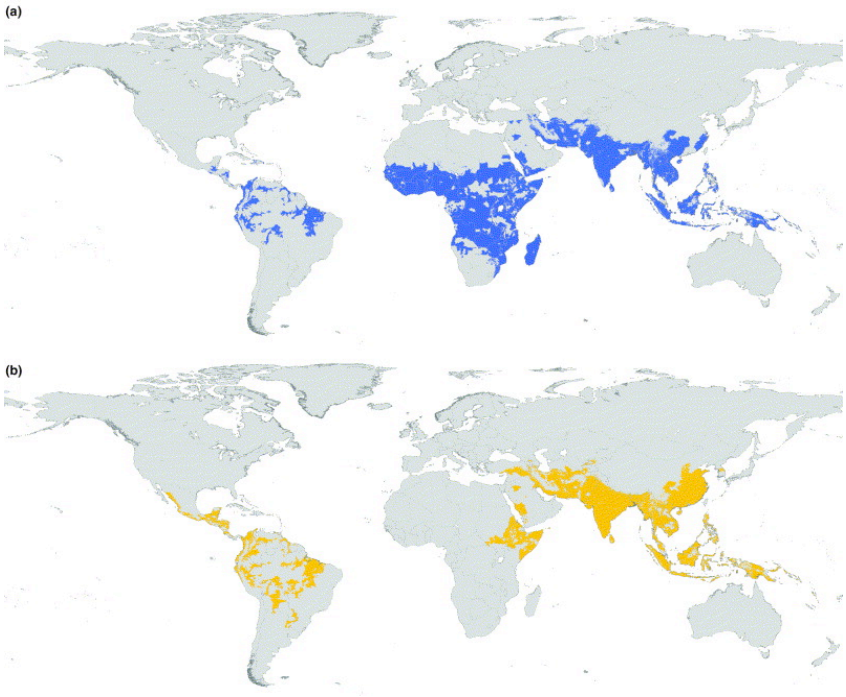

Figure 1 Global distribution of malaria due to (a) *P. falciparum* and (b) *P. vivax*. Adapted from (67) .

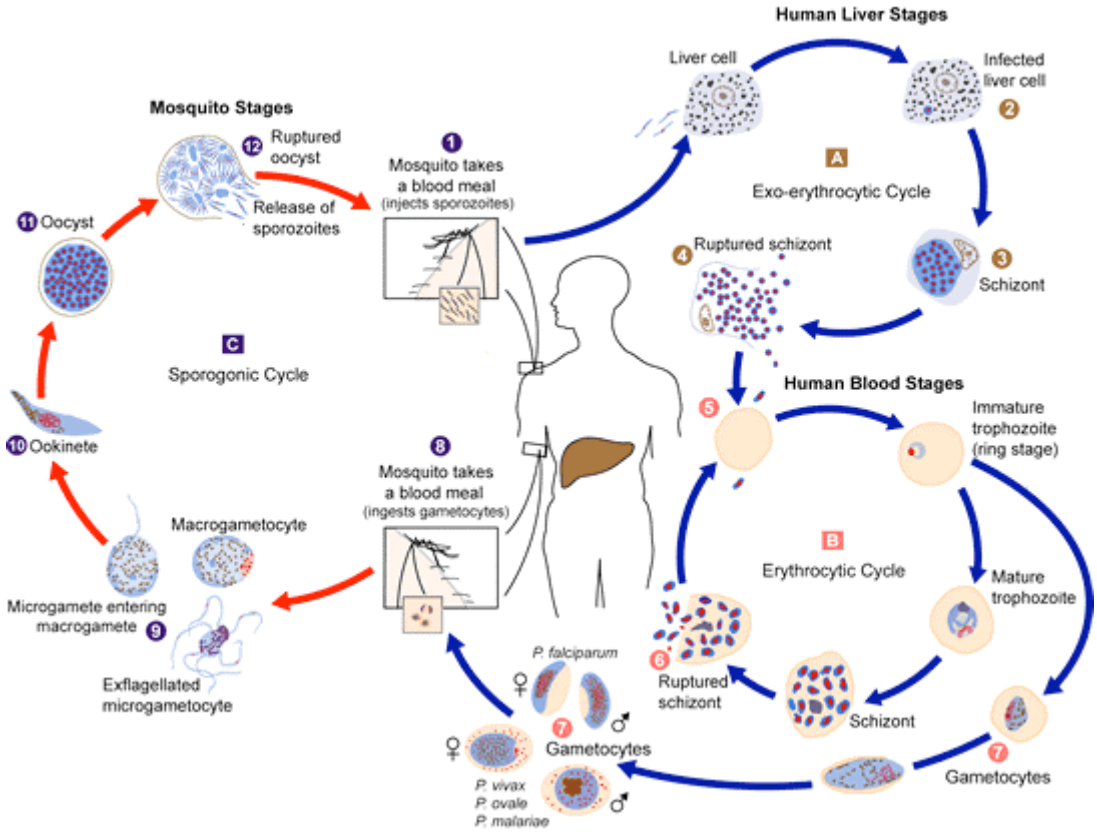

Figure 2. Life cycle of the malaria parasite. *Plasmodium* parasites are transmitted by the *Anopheles* mosquito, which infects a human while feeding, injecting sporozoites into the blood stream with the mosquito saliva. The sporozoites travel to the liver, where they infect hepatocytes and over the course of 7-10 days the parasite undergoes a series of nuclear divisions within the hepatocyte, replicating thousands of merozoites (135) which escape from the liver cell into the blood stream where they each invade a red blood cell, commencing the symptomatic course of the disease. The parasite cycle within the red blood cell lasts for approximately 24-72 hours (depending on species) and during this time, the parasite replicates DNA and organelles to create 12-32 new merozoites which burst out of the infected cell and invade new red blood cells. A small number of parasites do not continue to replicate in the red blood cell, but develop into either male or female sexual stages known as micro- and macrogametocytes, respectively. These circulate in the blood where they can be taken up by an *Anopheles* mosquito during a blood meal on the infected human. Once in the mosquito mid-gut, a number of factors, such as the decrease in temperature and the presence of xanthurenic acid, trigger differentiation of the gametocytes to gametes (15). The male microgametocyte rapidly undergoes three rounds of nuclear division to form eight microgametes, each of which can fertilise a female macrogamete, forming a zygote. This zygote will undergo one round of mitosis followed by one round of meiosis to form an ookinete, which traverses the mosquito midgut wall and develops into an oocyst. Nuclear divisions within the oocyst lead to the formation of thousands of sporozoites which are released into the mosquito haemocoel and travel to the salivary glands where they remain until the mosquito's next blood meal, infecting another human host, thus perpetuating the cycle (30). (Adapted from http://www.dpd.cdc.gov/dpdx)

1.2 *Plasmodium vivax:* A benign malaria?

P. vivax is traditionally known as the "benign" malaria due to the lower mortality compared to *P. falciparum.* As a result *P. vivax* has received less attention from policy makers and researchers in the attempt to eradicate malaria. Recently however, this view of *P. vivax* has been shifted to the realisation that this parasite is responsible for a serious disease that has been severely underestimated (111, 134). Due to its wide geographical distribution, *P. vivax* threatens 2.6 billion people (67) and estimates for the annual number of *P. vivax* infections range from 80 to a couple of hundred million (69, 134). These figures may even be underestimated due to inaccurate diagnosis of low level or mixed infections with light microscopy. The rapid diagnostic tests which are currently available may be unreliable in detecting low parasitaemias and cannot distinguish between *Plasmodium* species (190). Due to low transmission rates in *P. vivax* risk areas, protective immunity is rare and the disease affects all ages. Uncomplicated infections of *P. vivax* cause agonising disease which can persist for months if left untreated. In addition to acute episodes of high fevers, reports of severe disease attributed to monoinfection with *P. vivax* included cerebral malaria with seizures, hepatic dysfunction, lung injury with acute respiratory distress syndrome, renal failure, retinal haemorrhage, splenic rupture and severe anaemia (8, 96).

There are a number of features of *P. vivax* biology which must be considered if worldwide malaria eradication efforts are to be successful. The merozoites of *P. vivax* have a preference for infecting reticulocytes, immature forms of erythrocytes, which leads to severe anaemia despite relatively low parasitaemia.

P. vivax invades the red blood cell through interaction with the Duffy antigen on the surface of red blood cell, considered to be the only mechanism for invasion for *P. vivax*. A high proportion of the population of sub Saharan Africa are Duffy antigen negative and therefore *P. vivax* is

not common in these regions. However some recent reports suggest that *P. vivax* has infected Duffy negative individuals in Kenya and Brazil (25, 145) with confirmed *P. vivax* cases in Duffy negative individuals in Madagascar (110). If *P. vivax* evolves a new invasion mechanism which allows it to efficiently infect Duffy negative erythrocytes, it could quickly spread through Africa, creating a serious set-back for eradication efforts.

All blood stages of *P. vivax* can be found in the circulation of an infected patient. The parasite does not form "knobs" on the surface of the infected red blood cell and can therefore not adhere to endothelial cells and sequester in organs in a similar manner to *P. falciparum*. There have been reports of *P. vivax*-mediated damage due to apparent sequestration of the parasites in the lungs and spleen (6, 42). Given the inability of *P. vivax* to adhere to endothelial cells and sequester in the same manner as *P. falciparum*, these severe symptoms may be a result of a large inflammatory imbalance caused by *P. vivax* (5, 71).

Gametocytes of *P. falciparum* do not appear in circulation until seven to 15 days after the first blood stage cycle but gametocytes of *P. vivax* can appear in the peripheral blood of an infected individual even before the onset of clinical symptoms (111, 133). This means that an individual has the potential to transmit the parasite to a mosquito before realising that they are even infected.

The most exceptional feature of *P. vivax* is the parasite's ability to cause relapse weeks, months or years after the intial infection (87). This is due to the formation of dormant stages called hypnozoites in the liver of the host. A number of sporozoites do not develop immediately into schizonts once they have invaded the hepatocyte. They can remain dormant in the liver cell for many weeks before, due to unknown cues, they activate, replicating new merozoites and causing another blood infection (31). Strains of *P. vivax* from temperate regions tend to form more hypnozoites and remain dormant for longer than strains of *P. vivax* from tropical regions. This allows the parasite to survive periods of the year, such as

winter, when transmission is not possible due to the absence of the mosquito vector (10, 75). Primaquine is the only compound in clinical use which is effective against hypnozoites and thus prevent relapse of *P. vivax*. However, its mechanism of action is unknown, there are toxicity issues with the use of this drug, particularly in individuals with glucose-6-phosphate dehydrogenase deficiency where primaquine causes acute haemolysis and resistance to this drug may already have appeared (10). Hypnozoites represent a serious hurdle in the effort to eliminate malaria, primaquine will not be sufficient to address this issue, therefore efforts are underway to produce a 'radical cure' for *P. vivax*, which can purge the hypnozoites from the liver and prevent relapse (188).

1.3 Antimalarial drugs and the emergence of drug resistance

One of the major obstacles in the fight against malaria is the emergence and spread of *P. falciparum* strains resistant to all compounds in clinical use. A number of compounds are currently in clinical development, but the majority of these are derivatives of existing antimalarial drugs, or novel compounds in combination with an existing antimalarial (187), which could limit the efficacy of these treatments due to pre-existing, resistance-conferring mutations. This necessitates an understanding of drug resistance in the *Plasmodium* parasites in order to develop strategies to combat the onset of resistance and maximise the length of time these treatments remain effective.

The discovery of chloroquine for the treatment of malaria was hailed as the weapon which signified victory over the *Plasmodium* parasite, but the dissemination of chloroquine resistant parasites has rendered the drug all but useless. Extensive research is ongoing to understand the mechanism which parasites have developed to negate the drugs effect.

1.3.1. Chloroquine: mode of action and mechanisms of resistance

Chloroquine was once the wonder drug in the fight against malaria. Introduced in the 1940's, it was cheap to produce, was effective against the parasite blood stages and was associated with good tolerability and low toxicity. Extensive use of the drug worldwide led to the appearance of chloroquine resistant (CQR) *P. falciparum* in the 1950's in Thailand and Colombia, in the 1960's in Papua New Guinea and spreading to Africa by the 1970's (126, 185). Interestingly, CQR *P. vivax* did not emerge until the late 1980's in New Guinea but has since spread through parts of Central America, South East Asia and Africa (9). This surge in chloroquine resistance and the lack of convenient alternatives to chloroquine has led to a rise in the morbidity and mortality due to *P. falciparum*, particularly in Africa (177).

Chloroquine accumulates in the food vacuole of *Plasmodium* parasites and interferes with the crystallisation of haem into haemozoin (57). Chloroquine in its uncharged state is membrane permeable and distributes throughout the parasite, however once the drug enters the acidic environment of the food vacuole, it becomes trapped in its positively charged, membrane-impermeable form and accumulates to a concentration up to 20,000 times higher than the extracellular medium (147, 194). The protonated chloroquine molecule then forms complexes with haem, preventing the formation of beta-haematin dimers and their incorporation into haemozoin, leading to accumulation of toxic haem and death of the parasite (28, 57, 161, 173). Binding of chloroquine to haem may also account for a significant proportion of chloroquine accumulation in the *Plasmodium* food vacuole (20, 21). The basis of chloroquine resistance appears to be a reduction of levels of the drug at its site of action (21). CQR *P. falciparum* parasites accumulate four to ten times less chloroquine in the food vacuole than chloroquine sensitive (CQS) parasites (147). The presence of the calcium channel blocker verapamil

negates the resistance mechanism, restoring the response of CQR parasites to that of CQS (86, 107).

1.3.2. pH dependent changes

Initially it was proposed that the reduced accumulation of chloroquine in the food vacuole of CQR parasites was due to an increase in pH in the food vacuole (194). Considering the nature of the chloroquine molecule and according to weak-base theory, a higher food vacuole pH would result in lower accumulation of chloroquine (194). Two different methods were used by one group to measure the pH of food vacuoles in both CQS and CQR parasites: acridine orange or dextran-tagged probes. These studies suggested that food vacuole pH was in fact lower in CQR parasites compared to CQS parasites, which according to the Henderson-Hasselbalch equation, should result in increased chloroquine accumulation. (50, 178). They reasoned that a more acidic pH altered haem polymerisation kinetics; increasing the rate of haemozoin formation thereby decreasing the amount of haem available for chloroquine binding. The methods used in these studies have been called into question, due to the accumulation of acridine orange in compartments of the parasite other than the food vacuole and the buffering capabilities of the probes used which could alter food vacuolar pH (167), as well as the high sensitivity of the food vacuole to laser illumination during microscopic analysis (192). Two subsequent investigations, using either pH-sensitive fluorescent protein (89) or dextran-linked, pH-sensitive fluorescent dyes (70) have not found any difference in food vacuole pH between CQS and CQR parasites. Furthermore the structurally related quinoline antimalarials such as mefloquine and quinine are also amphipathic weak bases and if a change in pH was the determining factor in chloroquine resistance, then an altered pH would also modulate the response of the parasite to these drugs (167). On the contrary, CQR *P. falciparum* is often more susceptible to these drugs (22). It has become clear that while

modulation of pH in CQR may be a factor in determining chloroquine senstivity, it does not play a significant role.

1.3.3. PfCRT: Chloroquine transporter

The hypothesis that chloroquine is being transported out of the food vacuole by a carrier or transporter in CQR parasites, has been bolstered by a number of recent studies. A large body of evidence has been collected to implicate PfCRT as a facilitator of chloroquine transport across the food vacuole membrane and there remains little doubt that it forms a channel or pore through which, when the necessary mutations are present, chloroquine can pass.

A genetic cross between a CQS line and a CQR line of *P. falciparum* identified a single locus associated with inherited chloroquine resistance of 36kb on chromosome 7 (168, 186). Further investigations led to the identification of *pfcrt*, a 13 exon gene, predicted to code for a 424 amino acid protein with ten predicted transmembrane domains (56, 118). The protein encoded by *pfcrt* was expressed in asexual blood stages and was found to be localised to the food vacuole membrane (33, 56). The critical mutation which confers chloroquine resistance is the replacement of a lysine(K) at position 76 with a threonine(T) (43, 92) which is located in a predicted transmembrane region implicated in substrate recognition (105). The K76T mutation is ubiquitous in CQR parasites (43, 129). Accessory mutations in a further 14 positions always found in addition to K76T, depend on the geographical origin of the *P. falciparum* isolate and are expected to compensate for the K76T effect on the function of PfCRT (19). These additional mutations appear to also play a role in modulating the parasite response to other antimalarials (34). Allelic exchange experiments demonstrated that CQR alleles introduced into CQS parasites increased the IC50 of those parasites to chloroquine, which was verapamil reversible (157).

Although the endogenous function of PfCRT has not yet been defined, it has been identified as a member of the drug-metabolite transporter (DMT) superfamily, closely related to transporter proteins in *Cryptosporidium parvum* and *Dictyostelium discoideum* (105, 176). Plant homologs of PfCRT identified in *Arabidopsis* are located in the plastid and were shown to be involved in regulating glutathione levels in the plant cell cytosol (108).

Heterologous expression systems have proved useful in determining the role PfCRT plays in chloroquine transport. Recombinant PfCRT expressed in the yeast *Pichia pastoris* was shown to bind chloroquine (196) and when reconstituted into proteolipsomes was shown to increase chloroquine transport (174). Heterologous expression of PfCRT in the slime mould *Dictyostelium discoideum* demonstrated that mutant PfCRT which localised to the acidic endosomes, could expel chloroquine from these compartments (114). It was also noted in this study that the mutant form of PfCRT expressed in *D. discoideum* was associated a slight increase in endosomal pH, lending some support to the theory that modulation of pH may be important in chloroquine resistance (114). Mutant PfCRT expressed in the plasma membrane of *Xenopus laevis* oocytes transports chloroquine with higher efficiency than the wild type PfCRT (106).

The importance of the K76T mutation appears to be due to the charge within the channel. The change from positive (lysine) to neutral (threonine) allows the protonated chloroquine access to the channel. Verapamil may bind within the channel, restoring a net positive charge which repels the positively charged chloroquine (34). This has prompted a number of hypotheses to describe how PfCRT alters the accumulation of chloroquine in the CQR food vacuole, whether chloroquine travels through the channel in a passive, energy-independent process, or if PfCRT actively effluxes chloroquine out of the cell (20, 151).

1.3.4. Passive transport of chloroquine

PfCRT may function as a 'voltage-gated channel' where under energised conditions, the channel is open and allows the passive flow of chloroquine down its concentration gradient, out of the food vacuole and into the parasite cytoplasm (20). When the energy supply is removed or the proton gradient is reduced, the channel closes and blocks the flow of chloroquine out of the food vacuole (20). PfCRT has a degree of similarity to calcium chloride channels from a number of different organisms (183) and when PfCRT was expressed in *P. pastoris* and reconstituted into vesicles, it was responsible for a chloride-dependent decrease in pH inside the vesicles (196).

1.3.5. Active transport of chloroquine

An energy-dependent efflux was first suggested as the basis for chloroquine resistance when it was reported that addition of glucose to the medium of CQR parasites resulted in a decrease of chloroquine accumulation in the food vacuole while in contrast, additional glucose caused increased chloroquine accumulation in the food vacuole of CQS parasites (85, 86). This theory was further supported when it was demonstrated that this decrease in chloroquine accumulation in CQR parasites coincided with ATP production (150). This study also observed that chloroquine accumulation occurred in a *trans*-stimulated manner, where preloaded unlabelled chloroquine in the food vacuole of CQR, but not CQS parasites, stimulated the uptake of labelled chloroquine in the extracellular medium (150). *Trans*-stimulation is indicative of an active efflux mechanism rather than a passive diffusion, since movement of substrate through a channel in different directions blocks movement rather than enhances it. *Trans*-stimulated chloroquine efflux was demonstrated in both CQR and CQS parasites, where unlabelled chloroquine in the extracellular medium enhanced labelled chloroquine transport out of the food vacuole (149). In the presence of metabolic

energy, CQR parasites demonstrated a much higher chloroquine efflux rate than CQS parasites, which was verapamil sensitive (149). These transport kinetics were directly linked to the K76T mutation, which the authors proposed may allow binding of chloroquine to the carrier or alternatively facilitate coupling of chloroquine efflux to the proton-motive force (149).

1.3.6. Chloroquine resistance in *P. vivax*

CQR *P. vivax* was first detected in Papua New Guinea in 1989 (136), has spread throughout the Indonesian archipelago and appears to be expanding through South East Asia and South America (9, 111, 133). Chloroquine resistance took only ten years to appear in *P. falciparum* in a number of independent locations but chloroquine resistance strains of *P. vivax* did not appear for almost 40 years after introduction of the drug, despite heavy use throughout all endemic regions. One reason why chloroquine resistance took longer to appear in *P. vivax* may relate to differences in gametocytogenesis between *P. vivax* and *P. falciparum*. As mentioned previously, *P. vivax* gametocytes appear in the circulation of an infected individual very early in the course of the disease, meaning that the parasites can be transmitted prior to drug selection, reducing the selection pressure of chloroquine on resistant mutations. However, a limited number of studies suggest that chloroquine inhibits transmission of *P. vivax* gametocytes but not *P. falciparum* gametocytes (9, 23), which would serve to enhance the spread of chloroquine resistant *P. vivax.*

In *P. falciparum* chloroquine resistance can be diagnosed on recurrence of parasites in circulation following chloroquine treatment. Distinguishing between parasites present as a result of a recrudescence or reinfection can be done through genotyping. Characterisation of chloroquine resistance in *P. vivax* presents a challenge since reappearance of *P. vivax* parasites may be due to recrudescence, reinfection or relapse due to hypnozoite activation. For *P. falciparum* the only source of new infections

is from an infected mosquito bite, while new infections of *P. vivax* may be a result of an infected mosquito bite but also reactivation of a hypnozoite in the patients liver. Treatment efficacy is therefore evaluated based on plasma drug concentrations and the timing of recurrences (9). Treatment is deemed a failure due to chloroquine resistance if parasites reappear in circulation within 28 days of commencing treatment and if the whole blood concentration of chloroquine and desethylchloroquine (the major metabolite of chloroquine) of the patient on the day of parasite recurrence is 100ng/ml or greater (11).

Ex vivo assays also provide a practical means of assessing the response of *P. vivax* isolates to chloroquine, allowing objective comparison of chloroquine sensitivities of different isolates. Parasites are removed from the patient and after short-term culture *in vitro* in the presence of chloroquine, the parasite number is evaluated by either parasite lactate dehydrogenase assay, [^{3}H]hypoxanthine incorporation or microscopic examination (48, 144). The asynchronous nature of *P. vivax* infections means that the *in vitro* drug response of an isolate must be interpreted in light of the initial stage of the parasites and the duration of the assay. A further complication which has been recently highlighted by such *ex vivo* studies on *P. vivax* isolates is that *P. vivax* trophozoites appear to be intrinsically insensitive to chloroquine, in sharp contrast to *P. falciparum* (143, 154).

To date, no molecular markers have been found to correlate with chloroquine treatment failure in *P. vivax.* The *P. vivax* ortholog of PfCRT, PvCRT-*o* does not have any polymorphisms which are associated with increased resistance to chloroquine (9, 118). Heterologous overexpression of PvCRT-*o* in a CQS strain of *P. falciparum* increased the IC_{50} 2.2-fold (146). Expression of PvCRT-*o* in the slime mould *Dictyostelium discoideum* demonstrated reduced chloroquine accumulation in acidic endosomes in a verapamil-reversible manner (146). An engineered PvCRT-*o* sequence, which contained the K76T

mutation characteristic of CQR in *P. falciparum*, expressed in *D. discoideum* was also able to reduce chloroquine accumulation (146). This study serves only to demonstrate that PvCRT-*o* is capable of chloroquine transport, but considering the dearth of *pvcrt* mutations in CQR *P. vivax*, does not implicate PvCRT in chloroquine resistance in *P. vivax*.

These features of chloroquine effect and resistance in *P. vivax* warrant investigation into the presumed target of chloroquine, the food vacuole and characterisation of this organelle with respect to species specific differences which may highlight alternative modes of drug action.

1.4 The *Plasmodium* food vacuole: Haemoglobin uptake and degradation

During the asexual cycle in the erythrocyte, *Plasmodium* parasites ingest approximately 75% of the host cell cytoplasm, which is rich in haemoglobin (65). The ingested haemoglobin is transported to a specialized lysosome-like organelle called the food vacuole, or digestive vacuole, where is it degraded by a series of proteases, generating amino acids and haem, the iron-containing moiety of haemoglobin (65).

The most obvious function of haemoglobin degradation is to provide an amino acid source for the parasite, and indeed, labelled amino acids from haemoglobin are incorporated into *P. falciparum* protein (109, 156). However, haemoglobin is a poor source of methionine, cysteine, glutamine and glutamate and does not contain any isoleucine. Due to the parasite's limited ability for *de novo* amino acid synthesis (155, 175), it must rely on an exogenous source for these amino acids, particularly isoleucine. This is indeed the case; *P. falciparum* can grow well in medium containing only isoleucine, but these parasites have a much increased sensitivity to protease inhibitors, highlighting their dependence on haemoglobin degradation as a source of amino acids when amino acids in the medium are scarce (104). Naughton *et al.* have demonstrated that the inhibition of parasite growth when haemoglobin degradation is

blocked (by plasmepsin and falcipain inhibitors) is due to a block in protein synthesis (115).

However, not all the amino acids liberated from haemoglobin proteolysis are incorporated into protein. The parasite ingests ten times the amount of haemoglobin protein than it utilises as amino acids even in amino acid rich medium (88) indicating haemoglobin digestion is not only necessary for a source of amino acids. Some hypotheses propose that the parasite digests haemoglobin to make room to grow within the host cell (62), to maintain osmotic balance (97) and may use the amino acids as an energy source (155). The parasite may also use some of the liberated haem as an iron source (153).

Regardless of the primary role of haemoglobin degradation in the parasite, there is no doubt that this process is essential; if proteolysis of haemoglobin is blocked using protease inhibitors, parasite development is interrupted (59, 101, 104, 137-140)

1.4.1 Haemoglobin uptake and food vacuole formation

Early electron microscopy (EM) work on the feeding process of a number of *Plasmodium* species identified vesicles containing haemoglobin which orginate from double membraned endocytic invaginations on the parasite cell surface (141). These morpholgically distinct invaginations were termed "cytostomes" and appeared to become most active at the trophozoite stage (2). Further EM studies on *P. falciparum* extended this model to describe transport of the cytostomal-derived vesicles to the food vacuole where haemoglobin digestion takes place (162). An additional mechanism was observed which occurs in ring stages whereby small volumes of host cell cytosol were endocytosed by an cytostomal-independent process termed micropinocytosis (162). Haemoglobin degradation appeared to take place in these small vacuoles, as small crystals of haemozoin were visible before fusion of the vesicle to the main food vacuole (162).

Recently, improved imaging and molecular techniques have allowed more extensive study on this process in *P. falciparum* but has led to conflicting theories. Elliott *et al*. reported four different haemoglobin uptake mechanisms in *P. falciparum*. They proposed that a large phagocytic event in the ring stages, termed the "Big Gulp", leads to the formation of the food vacuole. Three additional uptake mechanisms are then responsible for the transport of haemoglobin to this compartment for processing. They described micropinocytosis events occurring throughout the cycle and phagotrophic vesicles which are formed only at the schizont stages (53). Cytostomal uptake had previously been perceived as the major contributor to haemoglobin uptake in *P. falciparum*, surprisingly however, this study claims that this process occurs only for a short period in trophozoites and is not responsible for the bulk of haemoglobin uptake (53). An independent study also using serial-section electron microscopy proposed a vesicle-independent mechanism of haemoglobin uptake whereby the cytostome invagination opposes the food vacuole and haemoglobin vesicles bud from the cytostomal tube directly into the food vacuole (94). However, results from other groups using methods complementary to EM agree with the model intially proposed by Slomianny (162) in which endocytosis of haemoglobin in *P. falciparum* commences in the mid ring stage via micropinocytosis, forming small haemoglobin containing vesicles which coalesce to form the food vacuole. The bulk of haemoglobin uptake subsequently occurs via the cytostome forming vesicles which are transported to the food vacuole for haemoglobin degradation (1, 44, 165).

It remains unclear if the haemoglobin containing vesicles derived from two different uptake pathways have different properties/capacities for haemoglobin degradation. Microcrystals of haemozoin have been observed in small vacuoles which appear to be derived from pinocytosis (44, 93, 119, 162). However, in larger vacuoles which are derived from the cytostome haemoglobin processing does not take place until reaching

the food vacuole. Furthermore, single membraned vesicles which contain haemoglobin have also been observed in the food vacuole, suggesting that for many vesicles degradation does not take place until delivery to the food vacuole (195).

Actin has been implicated at a number of points of the haemoglobin uptake process. Treatment of *P. falciparum* with actin inhibitors decreased cytostome number and impaired (but did not completely block) cytostome development (94). Actin inhibitors were also reported to cause the accumulation of undigested haemoglobin in the parasite, suggesting that actin is also required for transport of haemoglobin to the food vacuole, fusion of vesicles with the food vacuole or transport of proteolytic enzymes to the food vacuole (53, 165).

1.4.2 Haemoglobin degradation pathway in *Plasmodium*

The food vacuole contains a number of different types of proteases which are responsible for cleavage of the haemoglobin, to produce short peptides or amino acids which can then be transported out of the food vacuole to be incorporated into parasite protein or to be excreted. The different proteases which have been localised to the food vacuole and are probably involved in this process in *P. falciparum* include aspartic proteases called plasmepsins, cysteine proteases called falcipains, a metalloprotease called falcilysin and at least one dipeptidylpeptidase. Aminopeptidases must be involved but to date it is unclear if they are active in the food vacuole or in the cytoplasm.

Haemoglobin is degraded in a ordered process in which the plasmepsins make the initial cleavage of haemoglobin, making available cleavage sites for the falcipains and subsequently falcilysin, dipeptide aminopeptidases and aminopeptidases (65).

1.4.2.1 Aspartic proteases - plasmepsins

Four of the ten aspartic proteases, or "plasmepsins" in the *P. falciparum* genome are localised to the food vacuole, PfPM1, PfPM2, PfPM4 and PfHAP (histo-aspartic protease). All four genes are located at a single locus on chromosome 14 and appear to be products of a duplication event at some stage in the evolution of *P. falciparum* and *P. reichenowi*, since all other *Plasmodium* species sequenced to date have only one plasmepsin gene, an ortholog of PfPM4 (40). Transcripts for PfPM1 and PfPM4 peak early in the intraerythrocytic cycle while transcripts for PfPM2 and PfHAP peak in the latter part of the cycle, suggesting that these proteases function in specific roles at distinct stages of the asexual cycle (18, 95).

The substrate specificities of these enzymes indicate that they are responsible for the initial cleavage of native haemoglobin at position 33Phe-34Leu, which unravels the molecule and makes other sites available for processing by plasmepsins and other downstream enzymes (64, 66, 189, 193). The isoelectric points of the active plasmepsins (pH 4-5) are consistent with their role in the food vacuole (40).

Different parasite lines have been generated with single plasmepsin knock outs and in which different combinations of plasmepsins have been knocked out (16, 17, 103, 120). These knock out parasites had growth rates comparable to that of the parental strain except for a line in which *pm4* had been knocked out suggesting that this enzyme plays the most important role in haemoglobin digestion (103, 120). A triple knock out (*pfpm1, pfpm2* and *pfhap*) and even a quadruple knockout of all four plasmepsins was achieved, yielding viable parasites which had slightly slower growth rate than that of the parental strain in complete medium and produced slightly less haemozoin, clearly due to less efficient haemoglobin processing (16). This parasite line could not survive on amino acid limited medium, underlining its dependence on an exogenous

source of amino acids when the haemoglobin degradation process is impaired (16).

The intracellular targeting of PfPM2 has been studied in detail in *P. falciparum*. It is trafficked through the endoplasmic reticulum to the plasma membrane as a Type II membrane protein where it localises at the cytostome. The protein remains associated to the membrane until a cargo of host cell cytosol has been endocytosed and it is transported to the food vacuole in the endosome along with the haemoglobin (77). The *P. falciparum* plasmepsins are activated by cleavage at a processing site, conserved in all four proteins (124Gly-125Ser), to release the active enzyme into the lumen of the food vacuole. This activation step appears to be carried out by a falcipain, another food vacuole protease, although in the absence of a falcipain, the plasmepsins have a less efficient autocatalytic function (47).

1.4.2.2 Cysteine proteases – falcipains and vivapains

There are three cysteine proteases involved in haemoglobin digestion in *P. falciparum*, known as the falcipains: PfFP2, PfFP2' and PfFP3. All three have been localised to the food vacuole (38). Although all they share a high degree of identity, PfFP2 expression peaks earlier in the asexual cycle in early trophozoites, with FP2' and FP3 peaking in expression levels in late trophozoites and early schizonts (38). A bi-partite trafficking signal mediates correct targeting of the falcipains to the food vacuole. Two amino acids stretches, 16-25 in the cytoplasmic portion of the protein and 84-105 in the luminal domain, target the protein from the endoplasmic reticulum, through the Golgi to the plasma membrane and subsequent transport to the food vacuole in the cytostome-derived transport vesicles (170).

Falcipain-2 knock out parasites accumulate undigested haemoglobin in the food vacuole at the trophozoites stage, but this phenotype disappears by the schizont stage, presumably due to compensation by expression of

falcipain-2' and falcipain-3 later in the asexual cycle (159). Falcipain-3 could not be knocked out indicating an essential role for this enzyme (158).

Falcipain-2 and falcipain-3 do not appear to have a specific cleavage site in haemoglobin but display preference for smaller peptides with a leucine residue at position P2 of the cleavage site (169). This suggests that the falcipains are responsible for the bulk of haemoglobin hydrolysis, rapidly cleaving the molecule at multiple sites (169). Although the role of the plasmepsins in this process may not be essential, initial cleavage and unravelling of the haemoglobin molecule by the plasmepsins probably optimises the degradation process.

Orthologs of falcipains in *P. vivax*, the "vivapains" have similar but not identical substrate and inhibitor specificities to the *P. falciparum* falcipains (113). Vivapain-2 and vivapain-3 both hydrolyse haemoglobin at low pH although the pH optimum of vivapain-2 is less acidic and the enzyme appears to be more stable at neutral pH than vivapain-3 (113), suggesting that vivapain-2 plays a role in degradation of erythrocytic cytoskeletal components, similarly to falcipain-2 (49, 68).

1.4.2.3 Falcilysin

The peptides resulting from plasmepsin and falcipain action on haemoglobin are further processed by falcilysin. Falcilysin is a member of the M16 family of zinc metalloproteases and is localised to the food vacuole in *P. falciparum* (51). In acidic conditions similar to the *Plasmodium* food vacuole (pH 5.2), falcilysin has a preference for converting short polypeptides (approx. 20 amino acids) into oligopeptides of 5-10 amino acids. At neutral pH however, falcilysin more efficiently cleaves peptides rich in positively charged residues (112). Falcilysin has also been found to localise to the apicoplast and to a lesser extent, the mitochondria, where it is probably involved in

cleaving the targeting peptides of proteins trafficked to these organelles (132).

1.4.2.4. Processing of oligopeptides to dipeptides and amino acids

The dipeptidyl aminopeptidase DPAP1 is a cathepsin C homolog which was localised to the food vacuole in *P. falciparum* and releases dipeptides from the N-termini of haemoglobin-derived peptides (78).
It is unclear if terminal processing of short peptides/dipeptides to single amino acids takes place in the food vacuole or in the cytoplasm of the *Plasmodium* parasite. Two amino peptidases PfA-M1 and PfAPP were localised to the food vacuole in *P. falciparum* (39), however processing of haemoglobin oligopeptides by food vacuole extracts failed to yield any free amino acids (84). Aminopeptidase activity in the cytosol of the parasite has been demonstrated to degrade haemoglobin peptides (61) and a zinc aminopeptidase has been localised to a region in the parasite cytoplasm closely associated with the food vacuole membrane (4). It is possible that the final processing to amino acids takes place in both locations, depending on the availability and specificity of dipeptide and amino acids transporters which are present on the *Plasmodium* food vacuole membrane.

There is enormous redundancy within this degradative pathway in *P. falciparum*. Duplications have given rise to four plasmepsins, while other *Plasmodium* species express only one (40). The parasite is still viable in the absence of any of its plasmepsins and retains the capacity to degrade haemoglobin and produce haemozoin, relying on the three falcipains to compensate (16). There is an additional degree of redundancy in the processing of the proplasmepsins, they demonstrate autocatalytic activity in the absence of the falcipains (47). This redundancy presents a greater challenge to the development of

compounds which inhibit this process, but underlines the importance of this pathway to the parasite. The parasite simultaneously facilitates the formation of haemozoin from haem, another unique process which provides an opportunity for antimalarial development.

1.4.3 Haemozoin formation

A by-product of the proteolysis of haemoglobin is ferrous-protoporphryin IX, the iron-containing moiety of haemoglobin, which is rapidly oxidised to ferric-protoporphryin IX (FPIX) in the acidic environment of the food vacuole. FPIX or haem, is highly toxic to the parasite. At low concentrations (10-20μM), it is known to inhibit the plasmepsins and falcipains in the digestive vacuole (64, 181) as well as cytosolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (24, 55). FPIX production also generates highly reactive species such as hydrogen peroxide, superoxide radicals and the hydroxyl radical which mediate peroxidation of membrane lipids (13, 26, 79). In addition, haem is much more soluble in lipid than in water and can incorporate into the membrane, destabilising it (102) and making it more susceptible to hydrogen peroxide mediated lysis (29, 58, 121).

In order to prevent haem-mediated lysis, the parasite must abolish the toxic effects of the haem molecule. To this end, the parasite incorporates the haem into inert haemozoin, a dense crystalline substance which is visible by microscopic examination in the food vacuole of the parasite. This process in which *Plasmodium* forms crystals from large organic molecules is termed biocrystallisation (72) and represents a very efficient means of detoxification. Two haem molecules are covalently linked by an iron-carboxylate bond to form a β-haematin dimer which is then incorporated into the crystal lattice through hydrogen bonding (122). The

crystal is extended by continued incorporation of β-haematin dimers into the lattice.

1.4.3.1 Haemozoin formation: Protein or lipid mediated?

Given the hydrophobicity of haem, it seems logical that lipids may play an important role in initiating haemozoin formation and a number of studies support this theory (14, 45, 46).

Hempelmann *et al.* proposed that membrane lipids derived from the parasitophorous vacuole membrane transported along with the haemoglobin to the food vacuole may provide a scaffold for the developing crystal. The membrane lipid within the aqueous environment of the food vacuole could concentrate the hydrophobic haem and provide a nucleation centre for the β-haematin dimers (73). Pisciotta *et al.* observed that haemozoin crystals in *P. falciparum* were contained within "neutral lipid nanospheres" composed of mono- and diacyl glycerols in the food vacuole. They hypothesised that it is these neutral lipids which provide a hydrophobic environment that promotes formation of β-haematin dimers in the food vacuole (130).

The role of a protein in haemozoin formation was indicated by the ability of extracts of *Plasmodium* trophozoites to form β-haematin (161). The *P. falciparum* histidine rich proteins II and III (PfHRPII and PfHRPIII), the first candidates proposed for this role, were shown to bind haem and promote haemozoin formation (27, 152, 172) but lack of orthologs in other *Plasmodium* species (171) and the discovery of a *P. falciparum* lab clone which lacked both *hrpII* and *hrpIII* but was still able to produce haemozoin supported theories of lipid mediated haemozoin formation (117).

The Heme Detoxification Protein (HDP) has recently been identified as having haem binding properties and a very efficient mediator of haemozoin formation *in vitro*. The gene is very highly conserved across all

Plasmodium species sequenced to date and was not amenable to genetic disruption, suggesting an essential function for the protein. HDP was found to be localised to both the food vacuole of *P. falciparum* as well as the host cell cytoplasm and the investigators of the study proposed that the protein is secreted into the host cell and is then internalised along with the host cell cytoplasm by the cytostome and trafficked to the food vacuole (76).

This evidence suggests that haemozoin formation in the *Plasmodium* food vacuole occurs within lipid droplets where parasite proteins such as HDP may play a role in binding haem to chaperone or deliver it to the lipid spheres.

The unique nature of the *Plasmodium* food vacuole and the biochemical pathways involved in haemoglobin breakdown and subsequent haemozoin formation identifies it as an important target for new antimalarial compounds. Knowledge of this organelle in only one of the *Plasmodium* species may hinder the development of compounds effective against all species which cause malaria in man. At the very least, it must be a priority to characterise the food vacuole of the other *Plasmodium* parasite which is a major cause of human malaria, *P. vivax*.

1.5 *P. knowlesi*: a versatile model for *P. vivax*

Extensive cellular and molecular studies on the erythrocytic stages of *P. vivax* have been hindered due to the parasites narrow host cell specificity. *P. vivax* asexual stages have a strong preference for invading reticulocytes which cannot be maintained in *in vitro* culture. Therefore, studies with *P. vivax* are limited to *ex vivo* clinical isolates, where parasite load may be low and asynchronous and the parasites cannot be maintained for more than one or two cycles.

In the absence of an *in vitro* adapted *P. vivax* line, the primate malaria *P. knowlesi* represents a versatile tool for studying "vivax-like" biology. *P.*

knowlesi is a parasite of the long-tailed macaque (*Macaca fasicularis*) (30) is phylogenetically closely related to *P. vivax* (54) and is used for *in vivo* studies in its experimental host the rhesus macaque (*Macaca mulatta*).

P. knowlesi had been known to infect humans after experimental blood passage from a macaque to a human (80), but infections acquired naturally via mosquito bite were thought to be considerably rare. Molecular analysis of a number of malaria cases in South East Asia, has recently revealed that *P. knowlesi* is responsible for a much higher proportion of infections than had been anticipated (36, 160). These cases had been routinely misdiagnosed as *P. malariae*, since P*. knowlesi* is morphologically indistinguishable from *P. malariae* by microscopic examination. The 24 hour replicative cycle of *P. knowlesi* means the parasites may rapidly reach high numbers in the circulation of an infected individual causing severe symptoms (37). A clinical study in Malaysian Borneo classified one in ten *P. knowlesi* cases as severe, with 1-2% of cases leading to death (41).

P. knowlesi is the only *Plasmodium* species in addition to *P. falciparum*, which has been adapted to long-term, *in vitro* culture (82). The genome sequence of *P. knowlesi* has recently been published (123) and transfection technology has been developed for *P knowlesi* with both single- and double-crossover integration possible (83, 184). Its versatility in both *in vivo* and *in vitro* studies and the close phylogenetic relationship of *P. knowlesi* to *P. vivax* identifies it as an ideal model for characterisation of "vivax-type" biology.

2. Objectives of this thesis

This thesis describes the characterisation of a number of different aspects of food vacuole biology of *P. knowlesi*, using it as a closely related *in vitro* model of *P. vivax*. The versatile transfection technology of *P. knowlesi* is applied in a number of different ways to uncover differences between this primate malaria and the extensively studied *P. falciparum*.

Using different parasite staining techniques, in combination with imaging of *P. knowlesi* expressing fluorescently tagged food vacuole proteins, the morphology of the food vacuole is examined. Live confocal microscopy and electron microscopy are used to generate images of the parasites undergoing haemoglobin uptake and food vacuole formation.

The four plasmepsins in *P. falciparum* have been under scrutiny for a number of years as potential drug targets. The essentiality of the single plasmepsin expressed in *P. knowlesi* is investigated using a conditional protein expression system. Replacement of plasmepsinIV (*pkpm4*) of *P. knowlesi* with a functional copy tagged with a degradation domain, allowing control of protein levels in the parasite with the compound Shield. This system was used to study the growth rates of *pkpm4*-knock-down parasites in complete medium and amino acid-limited medium.

PfCRT is highly associated with chloroquine resistance in *P. falciparum*. The ability of this protein to modulate the chloroquine sensitivity in *P. knowlesi* is investigated by heterologous overexpression of CQS and CQR *pfcrt* alleles and the subsequent quantification of chloroquine susceptibility compared to wild-type *P. knowlesi*.

Collectively these studies give valuable insight into how similar/dissimilar this organelle and the biological processes which occur within it are between *Plasmodium* species, augmenting our knowledge and assisting the development of effective antimalarial drugs.

3. Materials and Methods

3.1. Generation of transfection vectors

Transfection constructs were generated according to standard molecular biology protocols (148). Molecular cloning enzymes and restriction enzymes were obtained from Invitrogen, unless otherwise stated. PCR amplification for cloning was carried out using Phusion DNA Polymerase (Finnzymes) with the protocol: three minutes at 98°C, 30 cycles of the following denaturation, annealing and extension steps: 30 seconds at 98°C, 30 seconds at 50-60°C (depending on primer melting temperature) and 90 seconds at 72°C, concluding with ten minutes of a final extension step at 72 °C. All primers are described in Table 1.

3.1.1. Fusion protein expression vectors

Fusion genes *pkcrt-gfp, pfcrt-gfp* and *pkpm4-mcherry* were generated in a double-amplification PCR fusion protocol similar to that described in Hobert (74). The first PCR amplification used Primer F (forward primer for the protein to be tagged) and the fusion primer (combines a reverse primer for the 5' end of the protein to be tagged with a forward primer for the fluorescent tag) with the gene of interest as a template, i.e. *pkcrt* was amplified from *P. knowlesi* cDNA. This product was amplified by PCR and checked for the correct size and sufficient yield on an agarose gel. The product from the first reaction acts as both a template and a primer in the second PCR reaction, combined with a reverse primer for the fluorescent tag and the forward primer from the first reaction. These are used in the second PCR reaction with the coding sequnce for the fluorescent tag, generating the full gene fusion. Product size and yield was verified on an agarose gel. The fusion was then cloned into the vector pCR-Blunt II-TOPO (Invitrogen) for sequencing and subcloning into the relevant expression vectors.

PkCRT-GFP and PfCRT-GFP expression vectors were generated from the vector Pk_pfcrt_HB3 (Figure 3A) which was provided by Dr. David Fidock, Columbia University. The fusion products *pkcrt-gfp* and *pfcrt-gfp* which had been cloned into pCR-Blunt II-TOPO were excised using the flanking XhoI sites (added during PCR amplification) and inserted into Pk_pfcrt_HB3 which had been digested with XhoI, generating a vector from which either *pkcrt-gfp* or *pfcrt-gfp* are expressed from the *pkcrt* promoter with a selection cassette containing the human dihydrofolate reductase (*h-dhfr*) selectable marker.

To generate the *pkpm4-mCherry* expression vector, the fusion gene *pkpm4-mCherry* (consisting of the first 91 codons of *pkpm4* fused to the mCherry coding sequence) was amplified with 750bp of the *pkpm4* upstream UTR and cloned into pCR-BluntII-TOPO (Invitrogen). This was then subcloned by BglII/XhoI digestion into BglII/XhoI digested Pk_pfcrt_HB3 to replace the *pkcrt* 5'UTR and *pfcrt* ORF with the *pkpm4* 5'UTR and *pkpm4-mCherry* fusion ORF. The orientation and sequence of the fusion genes in each of the expression vectors was confirmed by restriction digest analysis and sequencing.

3.1.2 PfCRT expression vectors

The expression vectors pDC_PfCRT_HB3 and Dd2trunc (Figure 3B) were obtained from Dr. David Fidock (56). pDC_PfCRT_Dd2full was generated by replacement of the truncated PfCRT Dd2 ORF in pDC_PfCRT_Dd2trunc with the full length PfCRT Dd2 cDNA from Pk_Pfcrt_Dd2 by XhoI digest. The orientation and sequence of the inserted *pfcrt* fragment was confirmed by restriction analysis and sequencing.

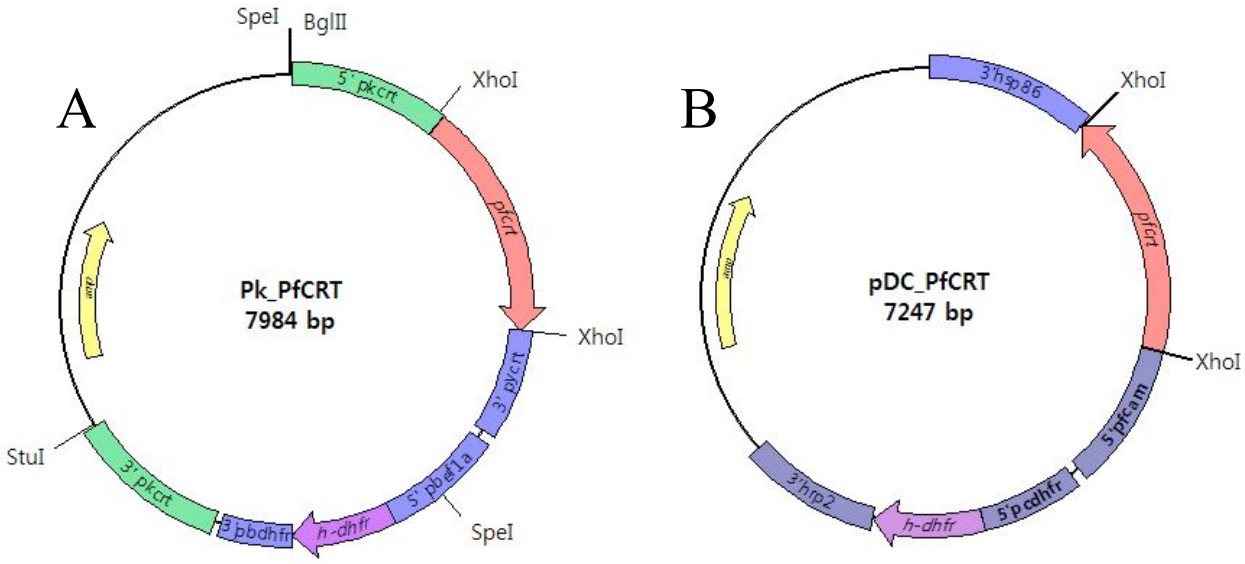

Figure 3. pDC_PfCRT vector and Pk_PfCRT vectors and restriction sites used for linearisation or cloning. A. Pk_Pfcrt replacement vector, *pfcrt*(red) represents either HB3 or Dd2 alleles. The vector can be linearised by BglII/StuI digest and should integrate by double cross-over into the *pkcrt* locus via the flanking regions homologous to *pkcrt* (green) replacing the endogenous *pkcrt* ORF with either the HB3 or Dd2 allele of *pfcrt* controlled by the *pkcrt* promoter. The *h-dhfr* under control of the constitutive *P. berghei ef1*α promoter allows for selection of transfectants with pyrimethamine. B. pDC_PfCRT expression episome. The *pfcrt* ORF (full-length HB3 or truncated Dd2 allele) is expressed under the constitutive *P. falciparum* calmodulin promoter. The expression of *h-dhfr* under the *P. chabaudi dhfr/ts* promoter bestows resistance to pyrimethamine.

3.1.3. *pkpm4* knock-out (KO) construct

The h-dhfr_TOPO expression construct consists of *h-dhfr* under control of the constitutive *P. berghei* elongation-factor 1 alpha (*ef1*α) promoter with cloning sites on either side of the selection cassette for insertion of homologous flanking regions to allow integration of the construct at a specific locus by double crossover (Figure 4). Flanking regions homologous to the *pkpm4* ORF or UTRs were amplified from *P. knowlesi* genomic DNA using primers with additional restriction sites (Table 1) and the products were cloned into pCR-Blunt II-TOPO. Different combinations of flanking regions were subcloned into h-dhfr_TOPO to generate three different KO constructs (Figure 4). The KO constructs consisted of the following combinations of homologous flanking regions: *pkpm4*KO1, up1

and dwn1; *pkpm4*KO2, up2 and dwn2; *pkpm4*KO3, up2 and dwn3. Each vector was linearised before transfection with the restriction enzymes EcoRI and EcoRV.

3.1.4 pkpm4DD replacement vector

The *Toxoplasma gondii* dihydrofolate reductase-thymidine synthase (*tg-dhfr/ts*) selection cassette was isolated from the plasmid b3D.DT^H.^D (kindly provided by Dr. Chris Janse, LUMC, Leiden, The Netherlands) by a HindIII digestion, overhangs were removed with Klenow Large Fragment DNA Polymerase (Invitrogen) and the cassette was inserted into the EcoRV site in pBluescript generating pBS_t-dhfr.

A 660bp terminator region from the 3'UTR downstream of the *P. yoelii* chloroquine resistance transporter gene was isolated from Pk_Pfcrt by XhoI/SpeI digest, blunt-ended and inserted into the blunt-ended EcoRI site in pBS_t-dhfr followed by the upstream insertion of SalI/NotI C-terminal destabilisation domain (DD) fragment excised from pTunerC (Clontech). The *pkpm4* ORF was amplified with primers pkpm4F and pkpm4R (Table 1), digested with BamHI and inserted into the BamHI site immediately upstream of the DD tag, generating a flanking region homologous to codons 256 to 451 of *pkpm4*, missing the stop codon and in frame with the DD tag. A region of the *pkpm4* 3'UTR 1353-2156 nucleotides downstream of the *pkpm4* start codon, was amplified with primers 3'pkpm4 flank F and 3'pkpm4 flank R (Table 1) and cloned into the EcoRV site downstream of the *tg-dhfr* selection cassette. Each point in the cloning procedure was verified by restriction digest analysis before proceeding to the next cloning step.

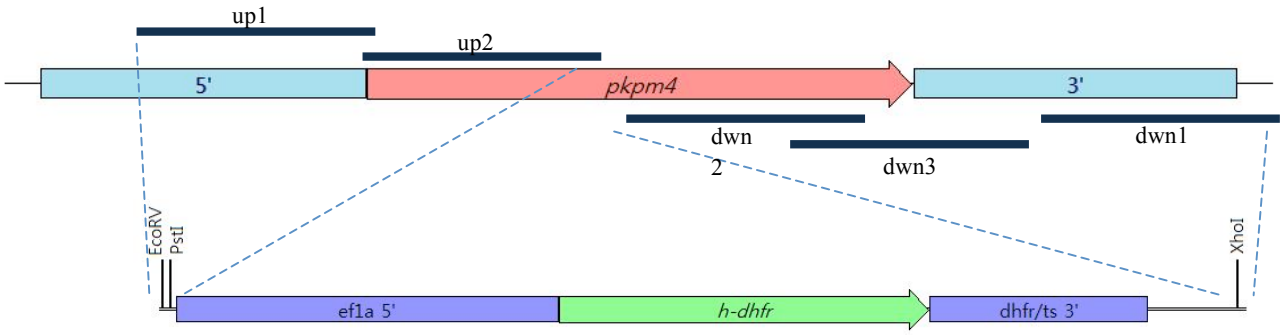

Figure 4. Integration vectors generated for *pkpm4* knock-out. A number of different regions of the *pkpm4* locus (top) were amplified by PCR and cloned in different combinations to flank the *h-dhfr* expression cassettte in hdhfr_TOPO (bottom), generating three different knock-out constructs. Each flanking region corresponds to the following sequence of the locus with respect to the start codon: up1, -648 to 32; up2, 1 to 842; dwn1, 2017 to 2796; dwn 2, 468 to 1330; dwn3, 933 to 1850.

3.2. *P. knowlesi* erythrocytic stages *in vitro* culture.

3.2.1 Routine *in vitro* culture of *P. knowlesi*

Parasites were cultured according to Kocken *et al.* (82). In brief, cultures were routinely kept at 5% haematocrit in rhesus macaque erythrocytes and complete medium (RPMI-1640, 20% heat-inactivated human serum, 15µg/ml gentamycin) in air tight culture flasks which were gassed with 5% CO_2, 5% O_2, 90% N_2 gas-mix and incubated at 37°C with gentle shaking.

The cultures were maintained at a parasitaemia of 0.1-2%, unless higher numbers were required for specific procedures when the parasitaemia was allowed to increase up to 12% with haematocrit reduced to 2%. Higher parasitaemias necessitated more frequent refreshing of the medium.

Cultures were routinely checked by Giemsa stain and the parasitaemia counted. To refresh the medium, the cells were spun at 750xg for 5 minutes at room temperature, the supernatant removed and fresh medium added with fresh rhesus erythrocytes if required, keeping the

period of time the parasites are at room temperature as short as possible. The culture was then gassed and replaced in the shaking incubator at 37°C.

3.2.2 Synchronisation – alanine treatment

To achieve a parasite culture in which all the parasites are at a similar stage in the cycle, it is necessary to carry out a synchronisation procedure. An alanine treatment results in a culture containing only young rings, the more mature stages have a more permeable host cell membrane and so are more susceptible to osmotic lysis. Five volumes of alanine solution (0.3M l-alanine, 1mM HEPES, pH 7.5) was added to one volume of cell pellet and incubated in a 37°C water bath for 20-30 minutes. The cells were washed twice with 20 volumes of RPMI-1640 and returned to culture.

3.2.3 Isolation of trophozoites and schizonts – Magnetic Cell Sorting (MACS)

The by-product of haemoglobin degradation, haemozoin is weakly magnetic due to its iron component. Mature asexual stages of the *Plasmodium* parasite accumulate this pigment as they digest more haemoglobin. The paramagnetic nature of these mature stages means that they can be purified from uninfected erythrocytes and younger ring stages which have not yet formed haemozoin (125).

A cell pellet of *P. knowlesi* trophozoites of approximately 500µl and at least 2% parasitaemia was washed once with RPMI-1640 and resuspended in MACS Buffer (2% bovine serum albumin in PBS, 2mM EDTA, pH7.5, filter sterilised).

A MACS CS-column (Miltenyl Biotech) was fitted to the Vario-MACS magnet (Miltenyl Biotech), and equilibrated with MACS Buffer for 10

minutes. The parasite suspension was then added to the top of the column in the magent and allowed to slowly filter through the column, until the parasite suspension had reached the bottom of the column. The tap was closed and the cell-filled column was incubated for 15 minutes at room temperature. The column was then washed with at least 30ml of MACS buffer, allowing the eluate to slowly drip out of the tap, until the eluate was free of cells. The column was removed from the magnet and flushed with 20ml of MACS buffer to collect the purified cells which were washed in RPMI-1640 and quantified in a haemocytometer and parasitaemia determined on Giemsa stained smears.

3.2.4 Percoll purification of schizonts

More mature asexual stages are less dense than the uninfected erythrocytes and can therefore be separated on a density gradient such as Percoll. 2.5ml of *P. knowlesi*-infected erythrocytes at 5-10% parasitaemia was washed twice in RPMI-1640 and resuspended to approximately 30% haematocrit, then gently applied to the top of a 7ml cushion of 52% Percoll in RPMI-1640 in a 15ml centrifuge tube, taking care not to disturb the interface between the Percoll and the cell suspension.

The gradient was spun at 1000xg for 20 minutes at room temperature with a low brake. The ring and trophozoite stages, along with the uninfected erythrocytes go through the Percoll and form a pellet, while the less dense schizonts remain at the top of the Percoll cushion. These were removed and washed at least three times in RPMI-1640 to remove the Percoll.

3.2.5 Cloning *P. knowlesi* pkpm4DD by limiting dilution

The number of parasites per millilitre was calculated by counting the cells/ml in a haemocytometer and the parasitaemia on Giemsa stained smears. The parasites were diluted to 100 parasites/ml in 5% haematocrit, and subsequently diluted to 2 parasites/ml in complete medium with 5nM pyrimethamine and 1μM Shield (Clontech). Four 96-well plates were filled with 100μl of the parasite suspension containing 2 parasites/ml, resulting in approximately 20% of the wells containing a single parasite. Ten wells were filled with 100μl of the 100 parasites/ml suspension for monitoring parasite growth. The plates were placed in a sterilised, air-tight container, which was gassed with 5% O_2, 5% CO_2, 90% N_2 gas mix, sealed and incubated at 37°C. The selection medium (complete medium, 5nM pyrimethamine, 1μM Shield) in the plates was replaced twice a week. After two weeks, smears of each well were made and Giemsa stained for screening. Any positive wells were screened for *pkpm4DD* integration by PCR on 2μl of cell pellet using primers 5' pkpm4 F and dd R (Table 1) and Phusion Blood II DNA Polymerase (Finnzymes) in the following cycling protocol: initial five minutes at 98C, 35 cycles of 98C for one second, 60C for five seconds and 72C for 30 seconds followed by a final extension step of 72C for one minute. PCR reactions were checked on an agarose gel and any isolates for which the PCR was positive were retained in culture for further genotypic analysis.

3.3. *P. knowlesi* transfection

3.3.1 Amaxa Nucleofector transfection

At least 5×10^7 schizonts directly from culture (synchronised by alanine treatment) or purified by either MACS or Percoll gradient, were resuspended in 100μl of Amaxa nucleofector solution (Lonza AG) in an Amaxa electroporation cuvette (Lonza AG). 5-10μg of plasmid DNA was

added and the electroporation was carried out in an Amaxa Nucleofector device (Lonza AG) using programme U-033. The electroporated mixture was immediately added to 10ml of complete medium with rhesus RBCs at 5% haematocrit, gassed and incubated at 37°C. 24 hours post-transfection, the medium was refreshed with complete medium containing 5nM pyrimethamine. The medium was subsequently refreshed every second day with regular screening of Giemsa stained smears to check for emerging transfectants. Pyrimethamine resistant parasites emerged 2-14 days post-transfection.

3.3.2 BioRad transfection

P. knowlesi were transfected in a protocol similar to that described in van der Wel *et al*. (180) with minor modifications. Briefly, 5×10^8 mature schizonts (≥6 nuclei) from synchronous *in vitro* culture were washed twice in RPMI-1640 and once in cytomix (120mM KCl, , 5mM $MgCl_2$, 0.15mM $CaCl_2$, 10mM KH_2PO_4, pH 7.6, 25mM HEPES, pH 7.6, 2mM EGTA, pH 7.6) then resuspended in 700µl of cytomix in a 4mm electroporation cuvette on ice. 50µg of linearised plasmid DNA was added, and the electroporation was carried out in a BioRad GenePulser II with Capacitance Extender at 2.5kV, 25µF, 200Ω (time constant 0.90). Following electroporation, the cell suspension was resuspended in 20ml of complete medium at 5% haematocrit, gassed and incubated at 37°C. 24 hours post-transfection, the parasites were washed once in RPMI-1640 and the medium was refreshed with complete medium containing 5nM pyrimethamine. The culture was refreshed every two days and regularly screened by Giemsa stained smears for emerging transfectants.

3.4. Genotypic analysis

3.4.1. *P knowlesi* genomic DNA isolation

Genomic DNA was isolated from *P. knowlesi* infected RBCs, using the MasterPure DNA Purification Kit for Blood Version II (Epicentre Biotechnologies). A red blood cell lysis step in Red Blood Cell Lysis Buffer, was followed by centrifugation to pellet the parasite cells, which were then lysed with Cell Lysis Buffer. The proteins were precipitated with Protein Precipitation Buffer, and pelleted by a centrifugation step. The genomic DNA was precipitated from the supernatant with cold isopropanol, and centrifuged at 13000xg at 4°C for 20 minutes. The DNA pellet was washed in 70% ethanol, dried and resuspended in TE Buffer and stored at 4°C.

3.4.2 Integration PCR

Integration of the pkpm4DD_tgdhfr construct was detected by PCR on genomic DNA isolated from the transfectant parasites using combinations of primers specific to sequences within the integration construct and sequences outside the regions of the locus used for homologous integration. To detect the presence of the intact wild-type locus, primers 5' pkpm4 F (P1) and 3'pkpm4 R (P2) were used. These sequences are present in both the wild-type and transfectant parasites, but the product of this reaction in the wild-type is 1.5kb, while the product in the transfectant parasites would expected to be approximately 7kb and too large to readily amplify by a standard PCR reaction, so should be absent in a PCR on genomic DNA from transfectant parasites. Integration of the construct was confirmed by PCR with primers 5' pkpm4 F (P1) and DD R (P3) to detect integration at the 5' end of the construct and primers tg-dhfr F (P4) and 3'pkpm4 R (P2) to detect integration at the 3' end of the integration construct (Table 1). The PCR reactions using recombinant Taq

Polymerase (Invitrogen) were as follows: three minutes at 94°C, then 35 cycles of the following denaturation, annealing and extension steps: 30 seconds at 94°C, 30 seconds at 56°C and 90 seconds and 72°C, concluding with ten minutes of a final extension step at 72 °C.

3.4.3 Southern Blotting

5µg of *P. knowlesi* genomic DNA was digested with HpaI (New England Biolabs) and separated on a 0.7% agarose gel. The agarose gel containing the separated DNA was exposed to UV light for 5 mins, denatured in 1.5M NaCl, 0.5M NaOH for 15 minutes at room temperature, neutralised in 1.5M NaCl, 0.5M Tris-HCl (pH7.5) for 15 minutes at room temperature and washed in 2× sodium chloride-sodium citrate (SSC). The DNA was transferred to a positively charged nylon membrane (Nytran SuPerCharge, Whatman) by capillary transfer in 2xSSC buffer overnight. The DNA was cross-linked to the membrane using programme C3 in a BioRad GS Gene Linker (150mJoules), the membrane was allowed to dry at room temperature and was stored at 4°C until hybridisation of probes.

The templates for probes were prepared by PCR using Phusion DNA Polymerase on the pkpm4DD integration plasmid using specific primers pkpm4 probe F, pkpm4 probe R, tg-dhfr probe F, tg-dhfr probe R (Table 1). The PCR products were purified and used as templates to generate DIG-labelled probes in an overnight reaction at 37°C using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche). Membranes were prehybridised in DIG EasyHyb (Roche) and the heat-denatured probe was added to a concentration of approximately 50ng/ml in DIG EasyHyb and hybridised overnight at 42°C. Low stringency (2% SSC, 0.1% SDS, 2x5 minutes at room temperature) and high stringency (0.5% SSC, 0.1% SDS, 2x15 minutes at 65°C) washes were carried out before blocking the membrane with Blocking buffer (DIG Wash and Block Buffer Kit, Roche), followed by addition of alkaline phosphatase(AP)-linked

antibody solution (1:10,000 in Blocking buffer) recognising the DIG label (Roche). After washing, the chemiluminescent substrate, CSPD (Roche) was added and the membrane was exposed to the chemiluminescent detector BioRad ChemiDoc XRS for 60 minutes and imaged using Quantity One 4.5.0 software.

3.4.4 Plasmid rescue

100ng of genomic DNA was transformed into competent *Escherichia coli* and plated on selective agar. Colonies which arose after overnight incubation at 37°C were cultured for mini-prep plasmid purification (148) and the purified plasmids were analysed by restriction digests.

3.4.5 Reverse transcriptase (RT)-PCR

mRNA was isolated from parasites using the Nucleospin RNA Blood isolation kit, according to the manufacturer's instructions. cDNA was generated from the parasite mRNA using random hexamers as primers and the reverse transcriptase in Superscript III First-Strand Synthesis Supermix (Invitrogen), according to the manufacturer's instructions. Specific *pfcrt* sequences were then amplified from the cDNA using recombinant Taq Polymerase (Invitrogen) and primer sets pfcrt rt-pcr F1 and R1 and pfcrt rt-pcr F2 and R2 (Table 1) which are specific for *pfcrt* but not *pkcrt*. PCR products were analysed by agarose gel electrophoresis.

3.5. Parasite imaging

3.5.1. Light microscopy

P. knowlesi (Hcc) and *P. falciparum* (NF54) were both *in vitro* cultured. *P. cynomolgi* (M strain) parasites were obtained from an infected rhesus macaque (*Macaca mulatta*) as part of an unrelated drug study. Images of *P. vivax* Chesson strain asexual stages came from an archived Giemsa-stained blood smear of *P. vivax*-infected *Aotus azarae boliviensis* erythrocytes (81). The Giemsa-stained smears were viewed with an Olympus BX41 light microscope and imaged with Olympus CellB imaging software.

3.5.2. Confocal microscopy

In vitro cultured *P. knowlesi* parasites were washed in Ringers Solution (122.5 mM NaCl; 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11mM D-glucose, 25 mM HEPES, 1 mM NaH_2PO_4, pH 7.4), then allowed to settle on a poly-l-lysine coated glass slide in a perfusion chamber. The parasites were incubated with 5μM LysoSensor Blue (Molecular Probes) or 1μM Hoechst (Molecular Probes) in Ringers solution, before a number of perfusions with Ringers Solution in the chamber to wash away excess stain.

The stained parasites were imaged using a Zeiss Axiovert 100M microscope with a 63x water-corrected lens and a Zeiss LSM510 confocal system (Zeiss, Germany) equipped with visible and UV laser lines. LS Blue was excited at 364 nm with the UV laser line and emission detected using a 385–470 nm bandpass filter. pHluorin and GFP were excited using the 488 nm Argon laser and the emission detected using an LP 505nm filter. mCherry was excited at 543nm with the Helium-Neon laser and the emission was detected using an LP 560nm filter. Images were analysed using ImageJ version 1.43 (NIH).

3.5.3. Electron microscopy

P. knowlesi parasites were isolated by MACS purification and fixed for electron microscopy according to the Tokuyasu technique. The cells were incubated in the first fixation buffer I (0.2M PHEM buffer, 8% paraformaldehyde, 0.2% glutaraldehyde) at room temperature for five minutes, followed by one hour incubation in fixation buffer II (0.1M PHEM (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM $MgCl_2$, pH 6.9), 4% paraformaldehyde, 0.1% glutaraldehyde) at room temperature. The cells were stored in 0.1M PHEM buffer with 4% paraformaldehyde at 4°C until proceeding to embedding. The fixed cells were washed in 50mM glycine in PBS before resuspension in 1% gelatin, followed by embedding in 10% gelatin. The gelatin was sliced into approximately 2mm cubes which were infused overnight at 4°C with 2.3M sucrose in PBS. Gelatin blocks infused with sucrose were mounted on cryo pins and frozen in liquid nitrogen. Ultrathin (90-120nm) sections were cut at -120°C with a Leica EM FC6 cryo-microtome and individual sections were placed on formvar-coated copper EM grids (Plano) in 1.15M sucrose, 1% methylcellulose/PBS. The grids were incubated in 2% gelatin/PBS at 37°C for 15 minutes, followed by incubation in PBS at 37°C for 15 minutes, washed in PBS, then extensively washed in distilled water on ice. The grids were dipped in 1.7% methylcellulose, 0.45% uranyl acetate solution and dried at room temperature. The grids were viewed with a Leica EM900 scanning electron microscope.

3.6. *In vitro* drug assays

The antimalarial activity of chloroquine was measured against *P. knowlesi* transfectants using the *Plasmodium* lactate dehydrogenase (*p*LDH) assay. This enzyme is produced by live parasites and is detectable in trophozoite and schizont stages. *p*LDH catalyzes the conversion of 3-acetylpyridine adenine dinucleotide (APAD) and lactate (both present in the substrate

buffer) to APADH and pyruvate. The enzyme diaphorase subsequently converts Nitro Blue Tetrazolium (NBT) (both also present in the substrate buffer) to Nitro Blue Formazan (NBF) using APADH as a reducing agent. NBF is a purple soluble substance that can be measured at wavelength 655nm. *P. knowlesi* cultures of 0.5% young rings which had been synchronised by alanine treatment in the previous cycle, were added to a two-fold dilution series of the drug (50mg/ml chloroquine phosphate, Bayer) from 0.057ng/ml to 116.25ng/ml in complete medium. The parasite cultures were incubated in flat-bottomed, 96-well plates at 37°C in the drug for approximately 40 hours (one and a half cycles), until the parasites had matured to the trophozoite or schizont stage, when the cell pellets were washed once in PBS and frozen at -20°C overnight. The LDH assay was carried out on the thawed cell pellets. 0.5mg/ml of NBT, 1U/ml diaphorase and 50µg/ml APAD were added to LDH buffer (100mM Tris-HCl, pH 8.0, 50mM sodium l-lactate, 2.5% Triton X-100) which was then added to the thawed cell pellet and incubated for 30 minutes, protected from light at room temperature with shaking. The optical density (OD) at 655nm of each well was measured in a BioRad 680 microplate reader. The OD values were used to calculate the %growth using the formula:

$$\%\text{growth} = 100 \times \left(\frac{(OD655^{\text{drugtreated}} - OD655^{\text{RBConly}})}{(OD655^{\text{nodrug}} - OD655^{\text{RBConly}})} \right)$$

Parasite inhibition values were plotted and statistical analyses performed using Graphpad Prism v5.0b.

3.7. Parasite growth rate assays.

Duplicate cultures of each clone were prepared in complete medium (RPMI-1640, 20% heat-inactivated rhesus serum (pooled from fasting animals), 15µg/ml gentamycin) one supplemented with 1µM Shield and one with no Shield added.

Custom produced RPMI-1AA (AthaenaES, Baltimore, USA) is similar to standard RPMI-1640 culture medium, but is deficient for all amino acids except isoleucine, which is present at the same concentration as in standard RPMI-1640 (50mg/l). Duplicate cultures were prepared in amino acid limited medium (RPMI-1AA, 20% rhesus serum, 15ug/ml gentamycin) also in the same shield concentrations.

The culture medium was changed every 24 hours (±1 hour), at the same time a thin smear was taken of each culture. The cultures were propagated simultaneously until the parasitaemia reached a limiting point (>3%). At the end of the assay, the smears of each culture from each time point were Giemsa-stained, blinded and the parasitaemia was calculated by counting a minimum of 5000 red blood cells. Growth curves were plotted and linear regression and statistical analysis were carried out using Graphpad Prism v5.0b.

Table 1. Primers used in the course of this research. Underlined sequences indicate restriction sites used for cloning.

Primer name	Sequence
Cloning	
Pkpm4-mCherry F	GTACATTCGAGCATTTTTAGAGTTATG
Pkpm4-mCherry fusion	CCTCCTCGCCCTTGCTCACCATCTTGTCATAGGGTCTCTCTA
Pkpm4-mCherry R (XbaI)	TCTAGATTACTTGTACAGCTCGTCCATGC
Pfcrt-gfp F (XhoI)	CTCGAGATGAAATTCGCAAGTAAAAAAAATAATCAAAAAAATTCAAGC
Pfcrt-gfp fusion	GAATAATTCTTCACCTTTAGACATTTGTGTAATAATTGAATCGACGTTGG
Pkcrt-gfp F (XhoI)	CTCGAGATGAAGATCTTGAAAAAGAAGAAAAAGGGGAAC
Pkcrt-gfp fusion	GAATAATTCTTCACCTTTAGACATTTGTGTTATTATCGATTCG
gfp R (BamHI)	CGGGATCCTTATTTGTATAGTTCATCC
pkpm4F	ATGGATATAGCAGTGAAAGAAC
pkpm4R (BamHI)	CGGGATCCCGTTCTTAGCGATAGC
3'pkpm4 flank F	TCGGAATGGTTAGGAGAAGAATC
3'pkpm4 flank R	CACTTATCGTTTGTTATTTTCTATC
Pkpm4KO up1 F (EcoRI)	GAATTCATCTGTTCGGCATTCGTGC
Pkpm4KO up1 R (PstI)	CTGCAGTGAGTAGTCTTGTTCTTTCACTGC
Pkpm4KO up2 F (EcoRI)	GAATTCATGGATATAGCAGTGAAAGAAC
Pkpm4KO up2 R (PstI)	CTGCAGGAAGGTAGAAGGTGAATAAGG
Pkpm4KO dwn1 F (XhoI)	CTCGAGGGTCTCTCCTTACTTTTAGCAC
Pkpm4KO dwn1 R (XhoI)	CTCGAG*GATATC*TTATTTCCTCAGTGTCCCCTTCG
Pkpm4KO dwn2 F (XhoI)	CTCGAGGACACTGGTTCAGCCAACTTGTGGGTCCCAAGTAAG
Pkpm4KO dwn2 R (XhoI)	CTCGAG*GATATC*CCGACACTCTCGTTGTCGTAGTCAAAAACG
Pkpm4KO dwn3 F (XhoI)	CTCGAGCACGACTTGTACTGGCAAATTGATTTAGATG
Pkpm4KO dwn3 R (XhoI)	CTCGAG*GATATC*GCCCGCTGAATCATGGTCGTTC
Checking *pkpm4DD* integration	
5' pkpm4 F (P1)	GTAACACTCATCCAGAAATACC
3' pkpm4 R (P2)	GTGGTAATAAGAAGATTTATCC
tg-dhfr F (P3)	TCCAGATGGAGATGGCTGTC
dd R (P4)	GAGATGGTTTCCACCTGCACTC
Amplification of templates for Southern blot probes	
Pkpm4 probe F	GGAAGGACAATGGATGACCGAACGG
Pkpm4 probe r	GCGTTCCTTGTTGAGGATGTTCACAATGG
Tg-dhfr probe F	GCATGGACAAATGACCCAAGGTTACT
Tg-dhfr probe r	CTTGTTTGCCTTGCAAGTGATATACATC
RT-PCR	
Pfcrt rt-pcr F1	TTCAAGCAAAAATGACGAGC
Pfcrt rt-pcr R1	GAGCACATTTACCAAGACAAG
Pfcrt rt-pcr F2	CGGTGATGTTGTAAGAGAACCAAG
Pfcrt rt-pcr R2	CGGAATCTTCATTTTCTTCATTTCTC

4. Results

4.1. Food vacuole morphology of *P. knowlesi*

In order to visualise and characterise the morphology of the *P. knowlesi* food vacuole, some specific features of the organelle were investigated. The presence of haemozoin is used as an indicator for haemoglobin catabolism. Low pH is another distinct attribute of the *Plasmodium* food vacuole, which can be used as a marker for the organelle. These characteristics were used in preliminary experiments to compare the food vacuoles of *P. knowlesi* to those of the well-characterised *P. falciparum*.

4.1.1. Haemozoin in Giemsa stained smears

Using the haemozoin pigment as a marker representing the food vacuole, the Giemsa stained slides were analysed for parasites throughout the asexual cycle (Figure 1).

In *P. falciparum*, the first visible signs of haemozoin are confined to a single location within the trophozoite (Fig. 3B), which continues to accumulate pigment due to ongoing haemoglobin degradation as the parasite matures (Fig. 3C, D). However, both *P. cynomolgi* and *P. vivax* trophozoites display diffuse scattering of small pigment crystals throughout the parasite cytoplasm (Fig.3J, N). As the parasites mature, these haemozoin crystals appear to coalesce into larger lumps (Fig. 3K, O) which eventually form a single mass of pigment in very mature schizonts (Fig. 3L, P). Scattered pigment crystals can also be seen in *P. knowlesi* trophozoites and early schizont stages (Fig. 3F, G), which similarly form a single large compartment containing haemozoin by the late schizont stage (Fig. 3H). In the trophozoite and early schizont stages, the pigment containing vacuoles often appear to be in close proximity to the plasma membrane.

Using visible haemozoin in Giemsa stained parasites as a preliminary marker for food vacuoles, this would suggest that *P. falciparum* is the

only parasite in this group which produces its haemozoin in a single compartment, the remaining species forming crystals of pigment throughout the parasite cell. Using this method to identify food vacuoles, the cells are fixed to a glass slide, making it impossible to determine if these pigment-containing compartments are discrete vacuoles, or if they are interconnected as part of a vacuolar network in the cell.

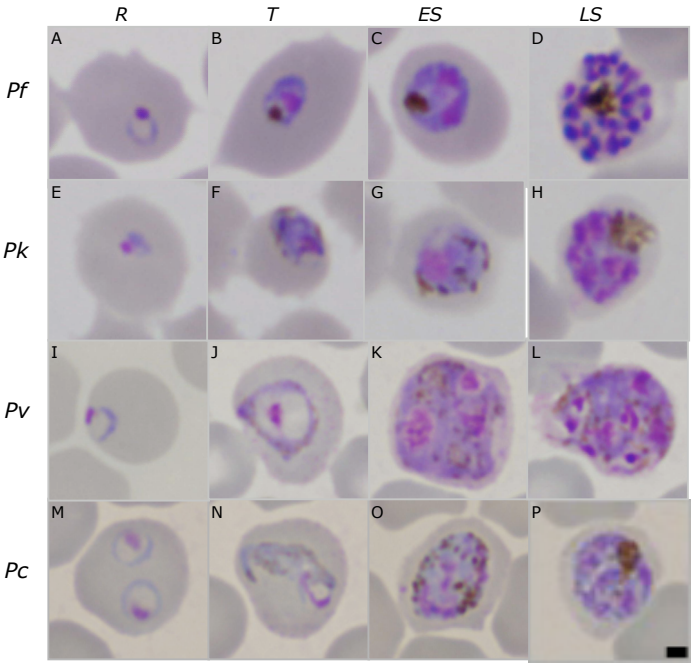

Figure 3. Giemsa stained smears of the asexual stages of human and primate *Plasmodium* species. Haemozoin is visible as brown lumps in the parasite. *P. falciparum* contains only a single mass of haemozoin. In the other species, haemozoin can be seen in lumps scattered throughout the parasite cell. *Pf*, *P. falciparum; Pk, P. knowlesi; Pv, P. vivax; Pc, P. cynomolgi*; R, ring; T, trophozoite; ES, early schizont; LS, late schizont. Scale bar 1µm.

4.1.2. LysoSensor Blue staining

In order to visualise the food vacuoles of live *in vitro* cultured *P. knowlesi* asexual stage parasites, the acidotropic stain, LysoSensor Blue was utilised. This dye accumulates and fluoresces in acidic organelles and has previously been used for food vacuole imaging in *P. falciparum* (192).

Live *P. knowlesi* parasites were stained with LysoSensor Blue and imaged using a confocal microscope (Fig. 4). A number of discrete compartments within the parasites could be seen which peaked in number in early schizonts (Fig. 4G) and appeared to coalesce as the parasite matured into a single large acidic vacuole, which contained a dense lump of haemozoin

(Fig. 4L). This is in contrast to LysoSensor Blue staining in *P. falciparum* trophozoites where a single acidic vacuole is visible (Fig. 4M). It was also noted that not all of the acidic vacuoles contain haemozoin, while not all the vacuoles containing haemozoin appear to be acidic (Fig. 4F, I). LysoSensor Blue staining was also carried out on live *ex vivo P. cynomolgi* parasites and viewed with a fluorescent microscope displaying numerous (>100), very small acidic vacuoles, consistent with the pattern of haemozoin in the parasites. Confocal imaging of stained *P. cynomolgi* was not possible and imaging with a normal fluorescent microscope was very difficult with the number and size of the vesicles.

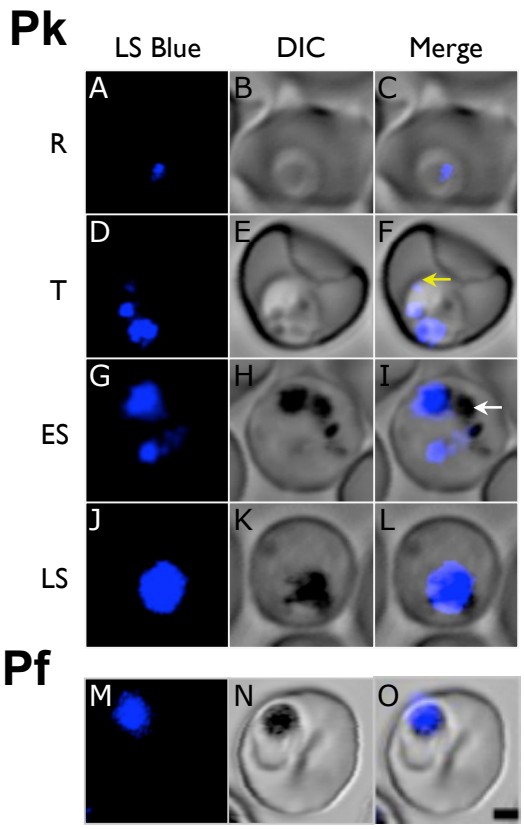

Figure 4. Live confocal imaging of LysoSensor Blue stained *Plasmodium* asexual stages. **Pk**. *P. knowlesi* asexual cycle. Trophozoites contain numerous acidic compartments which combine as the parasite matures to form a single acidic bacuole in late schizonts. Some acidic vacuoles do not appear to contain pigment (yellow arrow, panel F). Conversely, some pigment-containing vacuoles do not stain with LysoSensor Blue (white arrow, panel I). **Pf**. *P. falciparum* trophozoites contain a single acidic vacuole, in which a mass of haemozoin can be seen. R, ring; T, trophozoite; ES, early schizont; LS, late schizont. Scale bar 1μm.

Precise co-localisation of the LysoSensor Blue staining and haemozoin in *P. knowlesi* was made difficult due to the sensitivity of the food vacuole to light; the pH gradient is rapidly lost under illumination (192). Therefore an alternative approach was taken, using fluorescently tagged food vacuole proteins, as a more stable marker for food vacuoles.

4.1.3. Fluorescent tagging of food vacuole proteins

C-terminal fusions of the food vacuole proteins PkCRT (chloroquine resistance transporter) and PkPM4 (plasmepsinIV) to GFP and mCherry, respectively, were generated. The fusion proteins were cloned into expression vectors (Figure 3) under the control of the homologous promoter to allow correct stage expression and to avoid aberrant localisation due to overexpression and incorrect timing of expression.

The expression episomes were transfected into *P. knowlesi* using the Amaxa nucleofector system. Transfectant cultures were kept under pyrimethamine selection and resistant parasites emerged within 10 days, these live parasites were imaged with a confocal microscope. Loss of pH gradient due to illumination did not affect imaging of the fluorescent food vacuole proteins, allowing morphological studies in live parasites.

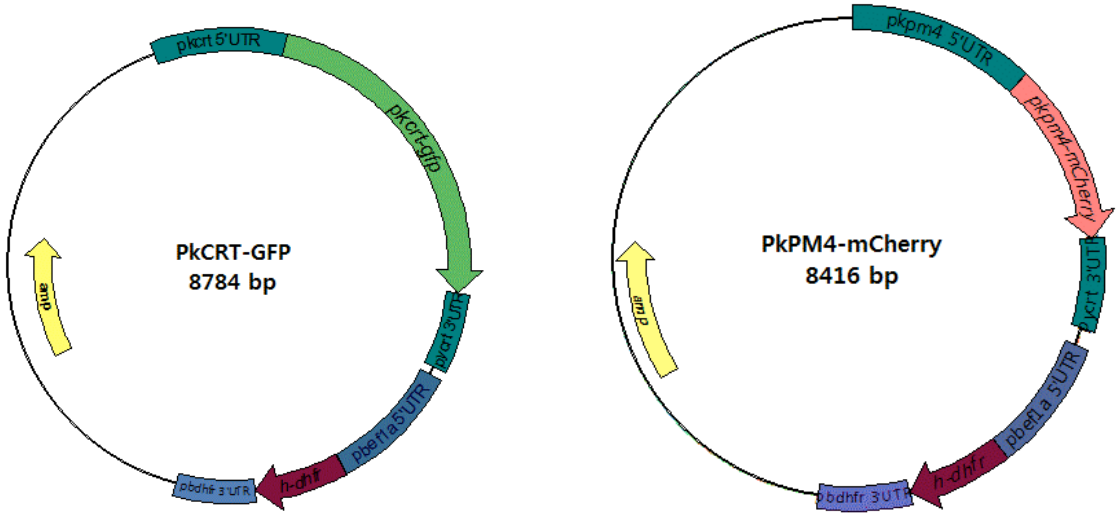

Figure 5. Expression episomes for fluorescently-tagged food vacuole proteins.

4.1.3.1. PkCRT-GFP

PkCRT is an integral membrane protein in which both the N- and C-termini are predicted to be exposed on the cytoplasmic face of the food vacuole membrane. The PkCRT-GFP fusion protein should indicate the location of food vacuole membranes within the parasite. The fluorescent signal in parasites expressing PkCRT-GFP (Fig. 6) appeared weak in the late ring stage in small points, which increased in number and signal strength as the parasites matured. The signal was most strong in mature schizonts when the food vacuoles have combined together to form a small number of larger vacuoles. It was only at the point where the parasite had formed one or two large vacuoles that the membrane location of the fluorescent tag was clearly distinguishable, the vacuoles in earlier stages were too small and too numerous to allow clear resolution of the membrane structure.

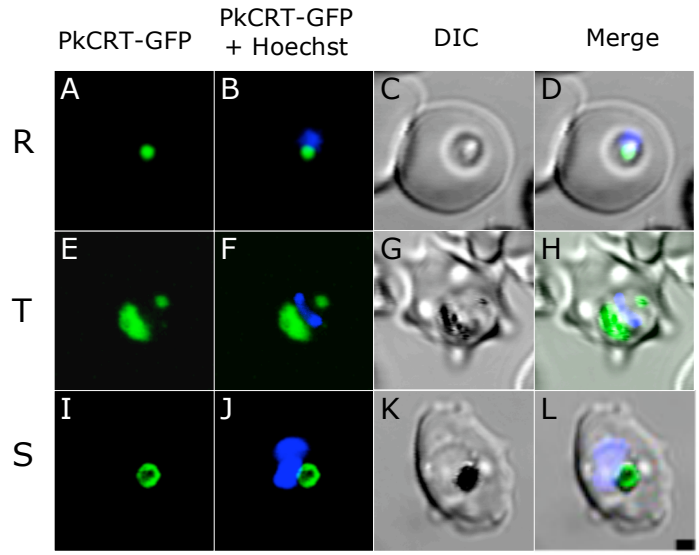

Figure 6. PkCRT-GFP episomal expression in *P. knowlesi* asexual stages. the fluroescent protein can be seen associated with haemozoin-containing vacuoles in trophozoites and schizonts. Scale bar 1µm.

4.1.3.2. PkPM4-mCherry

PkPM4 is trafficked to the parasite plasma membrane then is ingested with the haemoglobin on the membrane of the endocytic vesicles (12). Once the vesicle is acidified, the protein is cleaved from its transmembrane domain and released into the lumen of the vesicle. Thus,

PkPM4 with a C-terminal mCherry tag allows luminal staining of *P. knowlesi* food vacuoles. The signal from PkPM4-mCherry was clearly visible in transfectant parasites from an early stage in the asexual cycle (Fig. 7) and provided clear resolution of individual vacuoles.

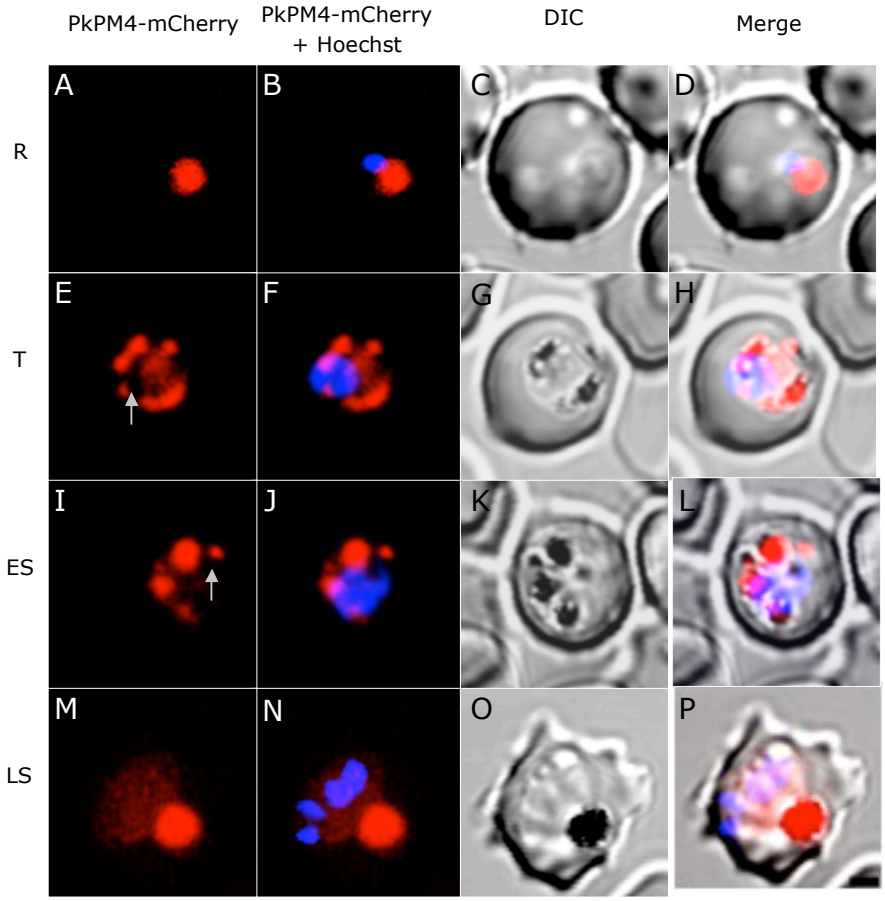

Figure 7. PkPM4-mCherry episomal expression in *P. knowlesi* asexual stages. Expressed from an early stage from the *pkpm4* promoter, this protein is clearly present in multiple vacuoles which also contain haemozoin. Vacuoles were observed in trophozoites and early schizonts with PkPM4-mCherry, but no visible haemozoin (grey arrows, panels E and I). Faint fluorescence is also visible in the daughter merozoites in the late schizont. R, ring; T, trophozoite; ES, early schizont; LS, late schizont. Scale bar 1µm.

PkPM4-mCherry positive vacuoles appear numerous in trophozoite and early schizont stages, often located at the periphery of the parasite cell adjacent to the plasma membrane in trophozoites. As the parasites mature, the vacuoles grow larger in size and smaller in number,

ultimately becoming a single vacuole as the parasite forms the daughter merozoites (Fig. 7M). A number of vacuoles were observed which contained the fluorescent PM4 protein, but did not have any visible haemozoin (arrow, Fig. 7F). A faint, dispersed signal of PkPM4-mCherry was also observed in the daughter merozoites in a number of segmented schizonts (Fig. 7M).

4.1.3.3. PfPM4-pHluorin

The expression vector pARL_pkpm4pHluorin was provided by Prof. M. Lanzer, which expresses pHluorin , a pH sensitive mutant of GFP fused to the N-terminus of PfPM4, which targets the fusion to the food vacuole (89). It was evaluated if *P. knowlesi* parasites expressing this fusion protein could be utilised to compare the pH of the individual food vacuoles and allow discrimination of separate vesicles from tubular networks. *P. knowlesi* parasites were transfected with the expression vector and successfully expressed the fusion protein which displayed a similar localisation pattern to the PkPM4-mCherry fusion protein (Fig. 7). Unfortunately, the vacuoles are too small and move too rapidly for accurate quantification of fluorescence intensity and pH comparisons could not be made.

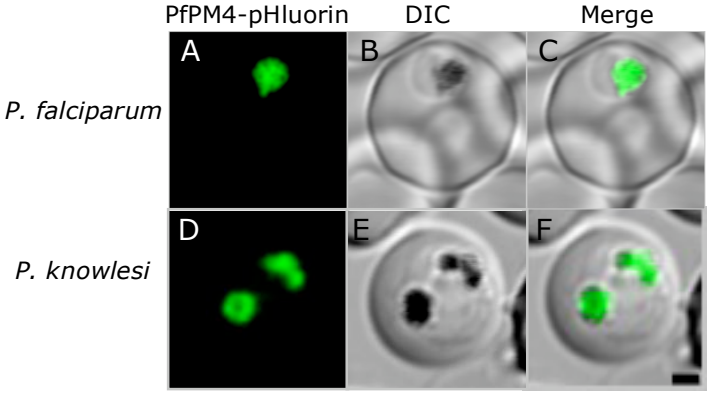

Figure 8. PfPM4-pHluorin episomal expression in *P. falciparum* (A-C) and *P. knowlesi* (D-F) erythrocytic stages. The flurorescent protein is visible in a number of different locations within the *P. knowlesi* parasite, while is confined to a single location in *P. falciparum*. Scale bar 1µm.

4.1.4. Co-localisation of acidic vacuoles and fluorescently tagged food vacuole proteins.

The nature of the individual vacuoles in *P. knowlesi* was further probed by LysoSensor Blue staining of *pkcrt-gfp* and *pkpm4-mCherry* transfectants (Fig. 9). LysoSenor Blue similarly stains multiple compartments of the transfectant *P. knowlesi* parasites, most of which are co-localised with the fluorescent food vacuole proteins. There is a small number of vacuoles however which are acidic, evident from the LysoSensor Blue staining, but do not appear to contain any of the fluorescent food vacuole marker protein, as seen most distinctly in the stained PkPM4-mCherry parasites (Figure 9G). These 2D studies obtain only the signal from the vacuoles within a slice of the parasite approximately 0.5-1μm thick, so it cannot be certain that the vacuole of interest has moved out of the focal plane between scans from the different lasers. It is possible that slight differences in the alignment of the microscope lasers fail to accurately quantify the fluorescent intensity in precisely the same position within the cell.

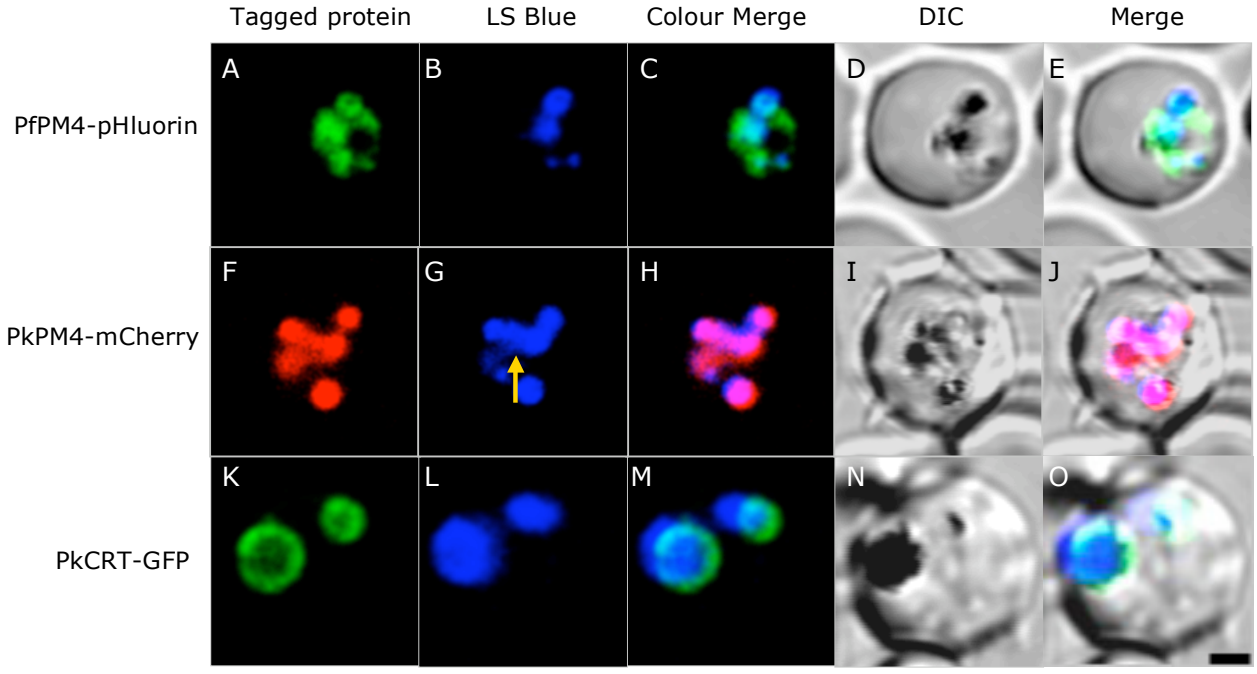

Figure 9. Live confocal imaging of LysoSensor blue stained transgenic *P. knowlesi* parasites expressing fluorescently tagged food vacuole proteins. Acidic vacuoles could be observed which did not appear to contain either haemozoin or a food vacuole marker protein (yellow arrow, panel G). Scale bar 1μm.

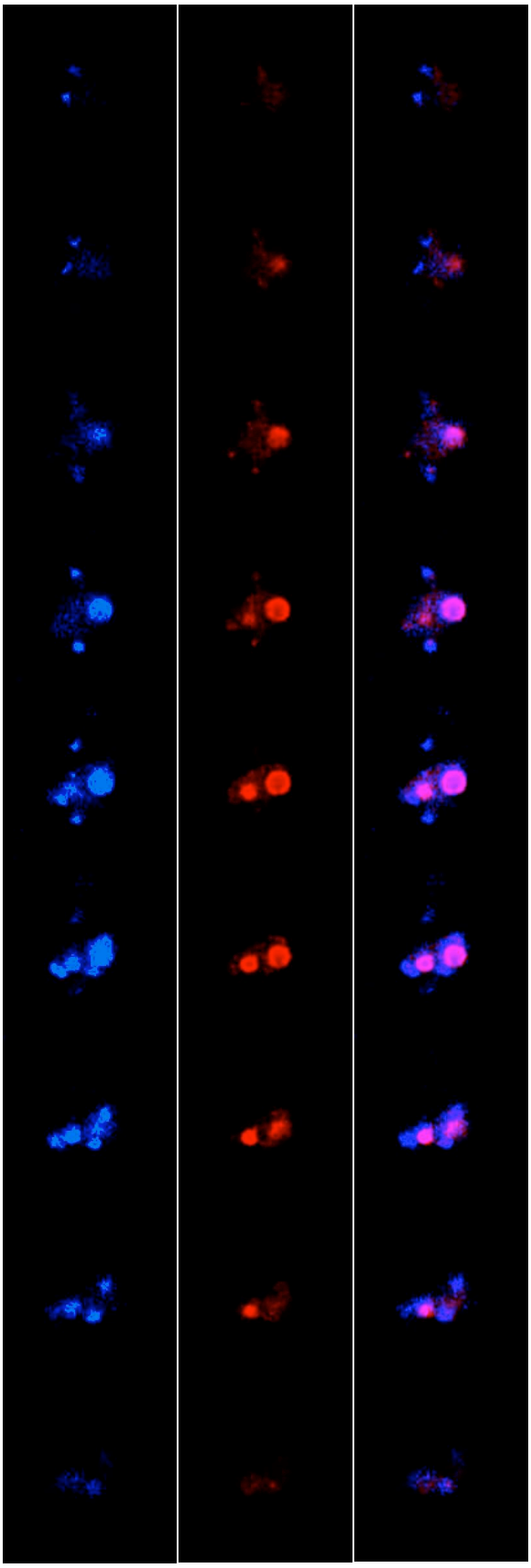

Figure 10. Series of z-stack images of LysoSensor Blue stained *P. knowlesi* expressing PkPM4-mCherry. Moving left to right represents slices of the cell from bottom to top. In slices 2-4 a number of vacuoles can be seen stained with LysoSensor Blue, but PkPM4-mCherry is absent.

To attempt more extensive mapping of fluorescent markers within the entire cell and possibly more accurate co-localisation of LysoSensor Blue and fluorescent food vacuole proteins, z-stacks through whole parasites were compiled. It was necessary to optimise settings for rapid scanning to capture signals for both channels from one slice as quickly as possible before vacuoles have moved too much, while maintaining adequate resolution.

Successive scans at intervals of 450nm from bottom to top through an entire *P. knowlesi*-infected red blood cell generated a series of images at different planes within the cell (Fig. 10). Each slice corresponds with the same plane of the cell for both mCherry and LysoSensor Blue. A number of different vacuoles are stained with LysoSensor Blue, most of which also contain PkPM4-mCherry. However it is evident from slices 2-4 that there one or two vacuoles which are stained with LysoSensor Blue but do not contain PkPM4-mCherry.

4.1.5. Electron microscopy of *P. knowlesi*

In order to visualise the smaller vacuoles of the haemoglobin uptake system in *P. knowlesi*, electron microscopy was carried out on MACS purified *P. knowlesi* trophozoites (Fig. 11). It is clear from EM images of *P. knowlesi* trophozoites, that haemoglobin is ingested by the parasite at multiple points along the parasite plasma membrane, where haemoglobin degradation takes place almost immediately, forming haemozoin. This is evident from the crystals visible in all the small vacuoles which are located along the inside of the parasite plasma membrane, complimenting the localisation of the vacuoles in LysoSensor Blue stained wild-type *P. knowlesi* and *P. knowlesi* expressing PkPM4-mCherry.

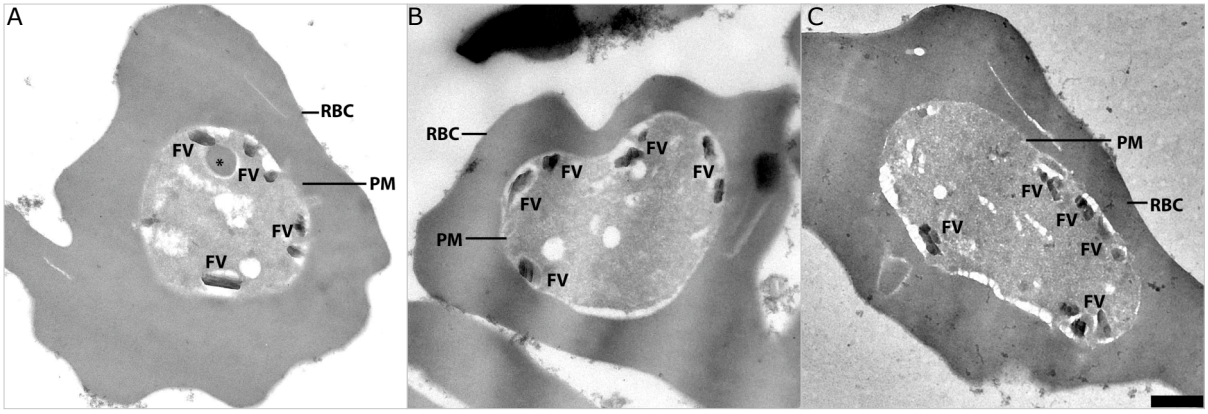

Figure 11. Electron microscopy of *P. knowlesi* trophozoites. Distinct compartments containing small haemozoin crystals (FV) can be seen along the inside on the parasite plasma membrane. In the parasite depicted in panel A, a region within the parasite can be seen (*) which has the same density as the host cell. RBC, red blood cell; PM, parasite membrane; FV, food vacuole. Scale bar 1µm.

P. knowlesi appears to contain multiple food vacuoles, in contrast to *P. falciparum* which forms a single acidic vacuole. The numerous acidic vacuoles were also observed to contain food vacuole marker proteins, with the exception of a number of vesicles which were stained with LysoSensor Blue but were not positive for PkPM4-mCherry, indicating that a small proportion of the acidic vesicles may not be functional food vacuoles.

4.2. *Pkpm4* knockdown using the FKBP DD system.

4.2.1 *pkpm4* knock-out.

A number of attempts were made to disrupt the *pkpm4* locus by double crossover integration using three different knock-out constructs with different sequences for homologous recombination (Fig. 4, Materials & Methods). *P. knowlesi* was transfected with the linearised constructs using the Amaxa transfection system. At least three knock-out attempts for each construct were carried out and pyrimethamine resistant parasites emerged in each case. Genomic DNA was isolated from emerging parasites and this was analysed by PCR for integration of the KO construct. None of the constructs were observed to have successfully integrated at the *pkpm4* locus, suggesting that the gene is essential for survival of *P.knowlesi* blood stage parasites or that technical issues with the Amaxa system may hinder efficient integration of the constructs. The attempt to knock-out the *pkpm4* gene was abandoned and an alternative approach was taken to investigate the essentiality of PkPM4 in *P. knowlesi*.

4.2.2. Knock-down of *pkpm4* using the FKBP destablilisation domain.

The FKBP DD system involves the tagging of a gene of interest with a destabilisation domain (DD) tag. Once translated, a protein carrying a DD tag is rapidly degraded within the cell. Addition of the compound Shield-1, which binds to the DD tag, stabilising the protein and protecting it from degradation, provides a means in which the levels of a tagged protein of interest within the cell can be controlled, allowing the study of protein function of essential genes. As the *pkpm4* gene was difficult to knock-out, this system was applied in an attempt to create a *P. knowlesi* parasite in which the expression levels of PkPM4 could be controlled and to determine if the locus was amenable to modification.

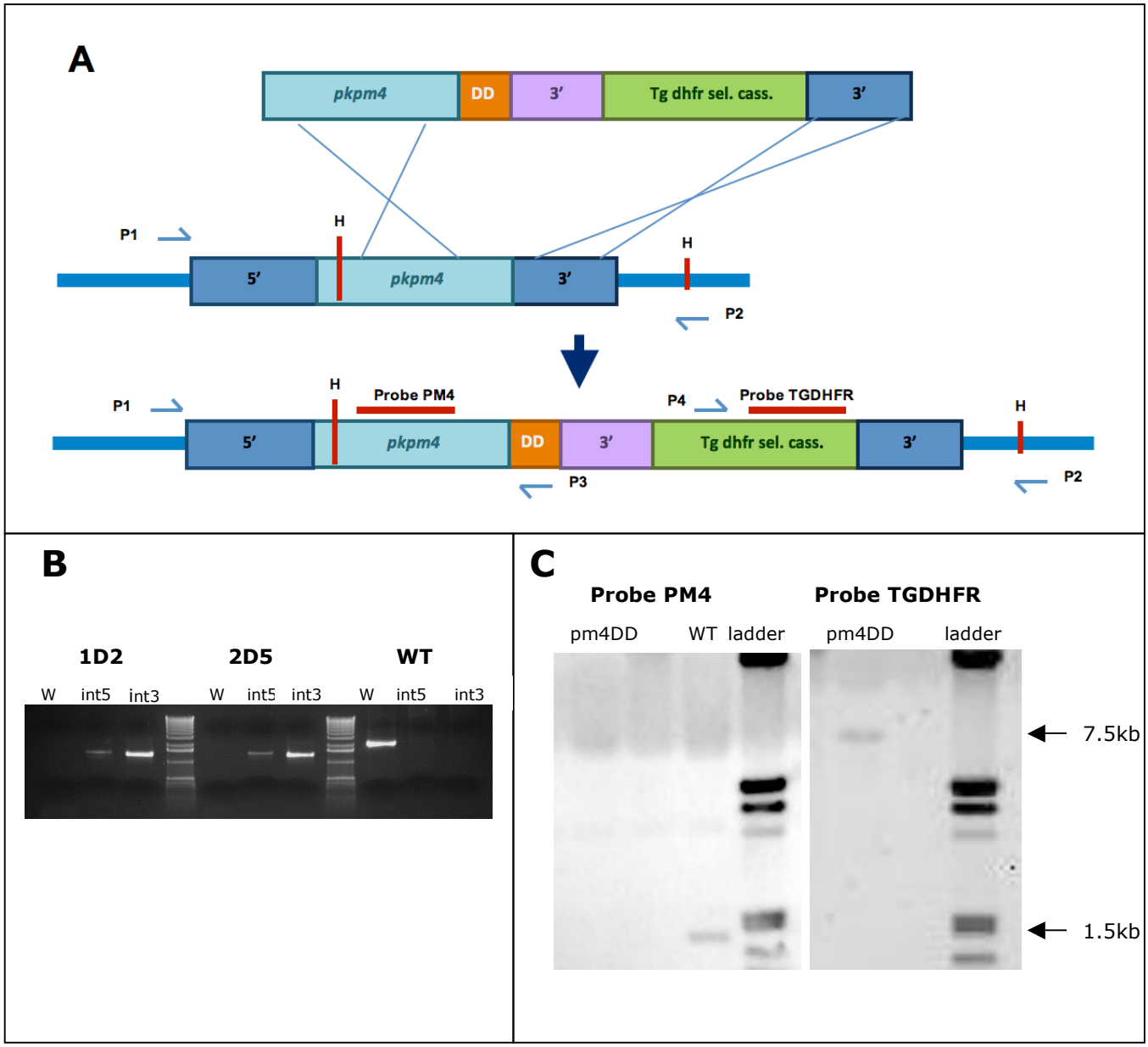

Figure 12. Replacement of *pkpm4* with the vector pkpm4DD. A. Integration strategy of pkpm4DD construct. Homologous recombination between the *pkpm4* ORF and 3'UTR and flanking regions in the construct results in double crossover integration generating a modified *pkpm4* locus, in which *pkpm4* has been replaced with a full-length *pkpm4* ORF in frame with a 3' DD tag, under the control of the endogenous *pkpm4* promoter. A *tg-dhfr/ts* expression cassette allows for selection with pyrimethamine. B. PCR confirmation of construct integration in pm4DD clones 1D2 and 2D5. Primer combinations for checking the presence of the wild-type locus (W), P1 and P2, integration at 5' end (int5), P1 and P3, integration at 3' end (int3), P4 and P2. The intact wild-type locus is absent from both clones, while positive results from both reactions int5 and int3 indicate successful integration. C. Southern blot analysis of recombinant *pkpm4DD* locus. *P. knowlesi* genomic DNA was digested with HpaI (restriction sites indicated by 'H' in A). A probe specific for a portion of the *pkpm4* ORF (Probe PM4) detects a 1.5kb HpaI fragment in wild-type *P. knowlesi*, but in pkpm4DD parasites the HpaI fragment is 7.5kb. This can also be detected with Probe PM4, but is partially obscured by a shadow on the blot. HpaI-digested transfectant DNA displays the same 7.5kb band when probed with Probe TGDHFR, specific to a region of the *tg-dhfr* ORF.

The linearised pm4DD integration vector (Fig. 12) was transfected using the BioRad electroporator into *P. knowlesi* schizonts (containing 6-8 nuclei). The culture was maintained under continuous pyrimethamine pressure to select for integrants in the presence of 1μM Shield-1 to stabilise the PkPM4DD protein. Pyrimethamine-resistant parasites emerged within twenty days. Parasites were cloned by limiting dilution, yielding clones 1D2 and 2D5. Genomic DNA was prepared and analysed by PCR and Southern blot confirming correct integration of the transfection construct and replacement of the endogenous *pkpm4* with a DD-tagged copy of *pkpm4* under the control of the homologous *pkpm4* promoter. (Fig. 12)

4.2.3. Appearance of WT and pkpm4DD parasites in response to Shield-1.

In complete medium, there is an excess of amino acids, so the parasites are not dependent on haemoglobin degradation as the sole source of amino acids. When free amino acids are absent in the parasite environment, the haemoglobin degradation pathway becomes vital to the parasite as an amino acid source. It is expected that parasites lacking a functional plasmepsin would be less efficient at haemoglobin degradation and thus display a distinct phenotype in amino acid-limited conditions.To generate culture conditions with very low levels of free amino acids, growth assays were carried out in RPMI-1AA supplemented with 20% rhesus macaque serum. Since isoleucine is not found in haemoglobin, it is present in RPMI-1AA at the same concentration as in standard RPMI medium. Initially, AlbumaxII was intended as a substitute for serum (similarly to the growth assays carried out on the *P. falciparum* quadruple plasmepsin knock-out, (16)) but RPMI-1AA supplemented with Albumax II would not support the growth of either wild-type or pm4DD *P. knowlesi* parasites in amino acid-limited medium. Instead, rhesus macaque serum isolated from blood taken from animals the morning after an overnight fast, was used to supplement the medium. Comparing the amino acid

concentrations in fasting rhesus serum (127), with free amino acid concentrations in RPMI-1640, the concentrations of all amino acids or their primary metabolites are 2-10 times lower in rhesus serum (with the exception of glycine, histidine, proline, tryptophan and valine which are present at comparable concentrations). The culture medium is supplemented with 20% rhesus serum, meaning that any free amino acids contained in the serum are found at concentrations 5-50 times lower in amino acid limited culture medium compared to complete culture medium.

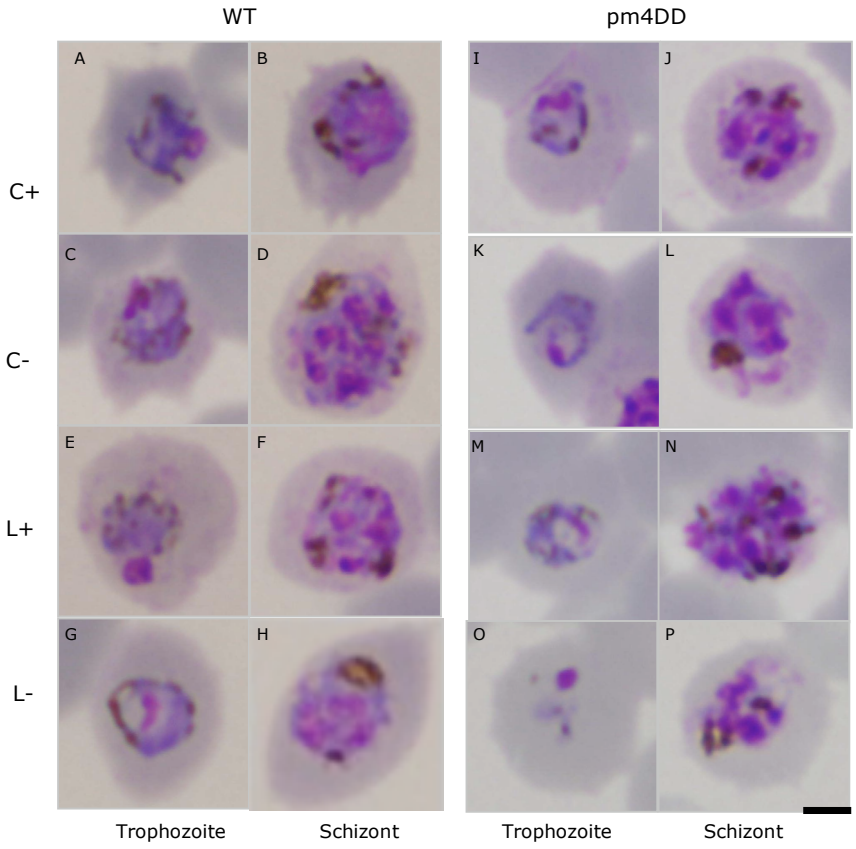

Figure 13. Giemsa stained *P. knowlesi* wild-type (A-H) and pm4DD (I-P) parasites in complete(C) and amino acid-limited(L) medium in the presence(+) and absence(-) of 1μM Shield-1. Scale bar 2μM.

The appearance of wild-type *P. knowlesi* appears unaffected by either Shield-1 or culture medium (Fig. 13 A-H). The appearance of trophozoites and schizonts of pkpm4DD parasites is unaffected by the absence of Shield-1 in complete medium (panels K and L), but in amino acid-limited medium, pkpm4DD parasites grown in the absence of Shield-1 (panels O and P) are much smaller and the cytoplasm of trophozoites and schizonts is hardly visible.

4.2.4. Growth rate of pm4DD parasites in response to Shield-1.

To investigate the importance of PkPM4 for intraerythrocytic *P. knowlesi* viability, the growth rate of pm4DD parasites was compared to that of wild-type *P. knowlesi* parasites in complete medium and amino acid-limited medium. Duplicate cultures of both *P. knowlesi* wild-type and pm4DD parasite were prepared in both complete and amino acid-limited media and one of each supplemented with 1μM of Shield-1. Cultures were propagated alongside each other and the parasitaemia was quantified by counting Giemsa-stained smears every 24 hours (± 1 hour) until the cultures reached a self-limiting parasitaemia (>3%). The parasitaemia of each culture on each day of growth was plotted to compare the effects of Shield-1 (Fig. 14). The growth assay was performed twice yielding similar results each time. The results of one assay are presented and are representative of both growth assays.

Pm4DD parasites always grow slower than the parental *P. knowlesi* parasites in each culture condition as expected. The growth of wild-type *P. knowlesi* is not affected by the absence of free amino acids in amino acid-limited medium (Fig. 14D). However, both pm4DD clones display a clear growth deficit in amino acid-limited medium compared to complete medium in the absence of Shield-1 (Fig. 14B, C, E, F). This growth deficit is partially alleviated for pm4DD parasites grown in amino acid-limited medium with 1μM Shield-1, but does not fully revert to the rate of pm4DD parasites in complete medium. Increasing the Shield-1 concentration to

3μM did not further improve the growth rate (data not shown) indicating maximal stabilisation of PkPM4DD occurs with 1μM Shield-1.

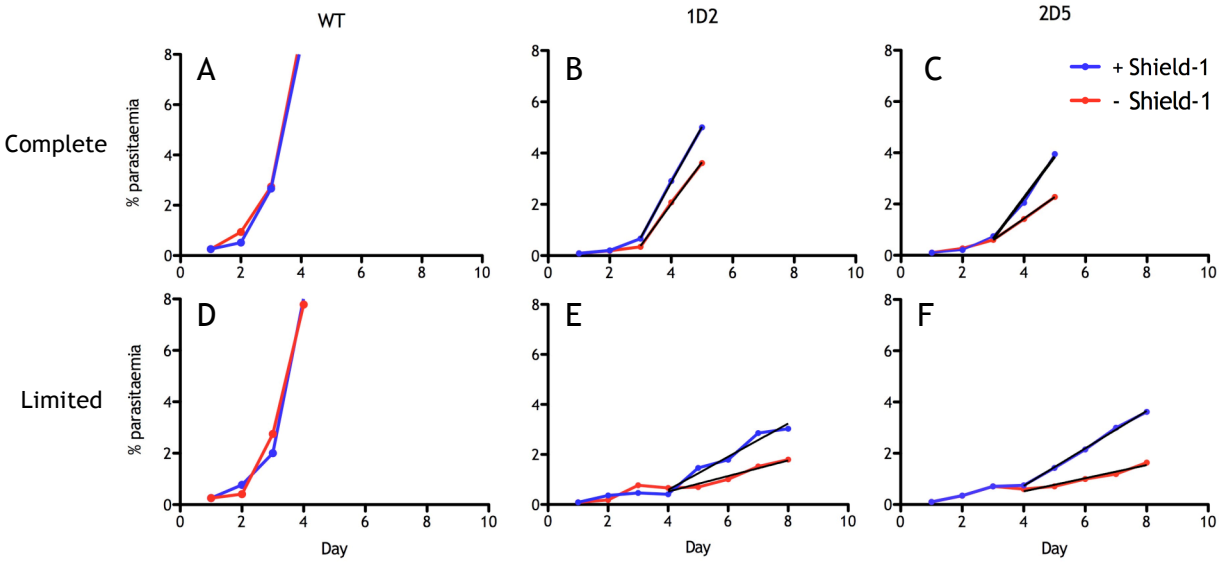

Figure 14. Growth rate of *P. knowlesi* wild type and pm4DD parasites in response to Shield-1 treatment. Wild-type parasite growth is not affected by either Shield-1 treatment or culture medium (A, D). Growth of Pm4DD parasites (clones 1D2 and 2D5) is much slower in amino acid limited medium (E, F) compared to complete medium (B, C). Black lines in panels B, C, E and F represent linear regression models of pm4DD growth rate.

To evaluate the effect of Shield-1 on pm4DD parasites the growth rate data were modelled by linear regression. Pm4DD parasites displayed a lag phase of three to four days, so the growth rates were analysed after this period. Parasite growth should be exponential which would be best described using non-linear regression, however due to the slow-growth of the pm4DD parasites, a linear model provided the best fit (black lines, Fig. 14B, C, E, F). In complete medium, the difference between the slopes of the linear models describing growth in the presence and absence of Shield-1 were found to be statistically significant for both pm4DD clones: 1D2, $p=0.020$; 2D5, $p=0.046$ (black lines in Fig. 14B, C). The effect of Shield-1 is found to be greater on pm4DD parasites in amino acid-limited medium as the difference between the slopes of the linear models is even

more statistically significant: 1D2, p=0.010; 2D5, p<.00001 (black lines Fig. 14E, F). Therefore the growth rate of pm4DD parasites in medium supplemented with 1μM Shield-1 is significantly higher than the growth rate of pm4DD parasites without Shield-1 in both complete and amino acid-limited media, strongly suggesting that Shield-1 is protecting PkPM4DD from degradation.

4.3. PfCRT in *P. knowlesi*

4.3.1 Heterologous replacement of *pkcrt* with *pfcrt* in *P. knowlesi.*

To determine if PfCRT was capable of modulating the chloroquine sensitivity of a non-falciparum *Plasmodium* parasite, closely related to *P. vivax*, which has never been under chloroquine selection, it was attempted to replace the *P. knowlesi* CRT homologue *pkcrt*, with either a CQS or CQR allele of *pfcrt*.

Replacement constructs to integrate at the *pkcrt* locus by double crossover integration were kindly provided by Prof. David Fidock (Columbia University) (Fig. 3A, Materials and Methods). *P. knowlesi* schizonts were transfected with the linearised replacement vectors using the Amaxa transfection system and selected for with pyrimethamine. The contructs should integrate by double crossover at the *pkcrt* locus via the homologous flanking regions 5'pkcrt and 3'pkcrt, replacing the endogenous *pkcrt* with either the HB3 or Dd2 *pfcrt* allele and a *h-dhfr* expression cassette. Pyrimethamine resistant parasites emerged but genotypic analysis by PCR indicated that the *pkcrt* locus remained intact. After four successive failures, the replacement of *pkcrt* with *pfcrt* was abandoned.

4.3.2 Overexpression of *pfcrt* in *P. knowlesi* from an episome.

It has previously been demonstrated that overexpression of *pfcrt* from an episome in a CQS *P. falciparum* parasite could modulate the chloroquine sensitivity of that parasite (56). Since *pkcrt* could not be replaced with *pfcrt* in *P. knowlesi*, the episomal expression constructs used in *P. falciparum* were obtained from Prof. David Fidock, to investigate if PfCRT expressed *in trans* could modulate the chloroquine sensitivity of *P. knowlesi*. pDC_PfCRT_HB3 and pDC_PfCRT_Dd2trunc constructs described in Figure 3B (Materials and Methods), were transfected into *P. knowlesi* using the Amaxa Nucleofector system. Under pyrimethamine

selection, transfectants emerged within seven days. Drug assays with chloroquine were carried out on the transfectant parasites alongside wild-type *P. knowlesi*. Growth inhibition curves representing at least three independent drug assays of *P. knowlesi* pDC_PfCRT_HB3 and pDC_PfCRT_Dd2 parasites treated with chloroquine (Fig. 15) and corresponding IC_{50} values (Table 2), display unaltered chloroquine sensitivity compared to untransfected *P. knowlesi*. Plasmid rescues, followed by restriction analysis and *pfcrt* sequencing confirmed the presence of the correct vectors with the expected *pfcrt* sequence in each transfectant line. RT-PCR with primers specific for *pfcrt* confirmed that transcripts of both HB3 and Dd2trunc *pfcrt* were present (data not shown).

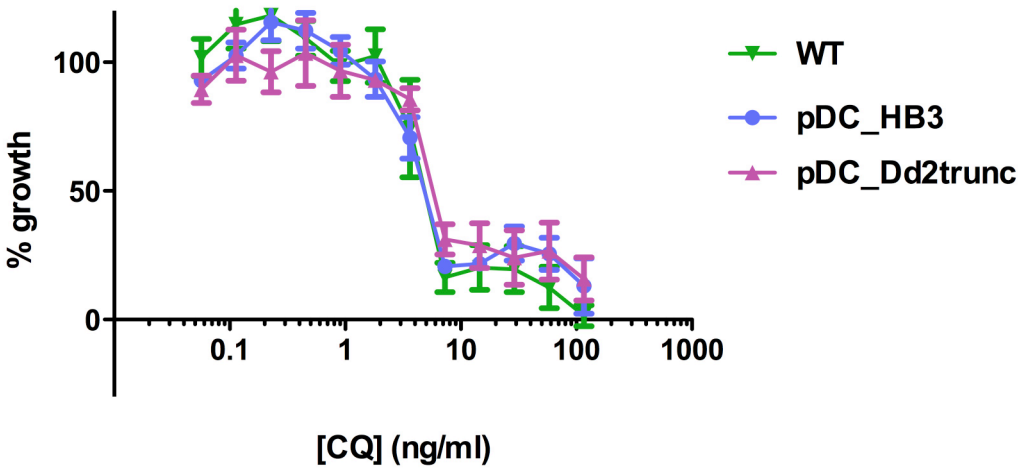

Figure 15. Growth inhibition curves of *P. knowlesi* (green), and *P. knowlesi* transfectants pDC_PfCRT_HB3 (blue) and PDC_PfCRT_Dd2trunc (pink) presented as mean±standard error. All lines were tested in triplicate in three independent assays.

4.3.3. Localisation of PfCRT in *P. knowlesi.*

The PfCRT Dd2 allele expressed from pDC_PfCRT_Dd2trunc was truncated by ten amino acids at the C-terminal. A recent study reports that the threonine residue at position 416 in PfCRT is essential for correct targeting of PfCRT to the food vacuole in *P. falciparum* (90). The *pfcrt* protein expressed from pDC_PfCRT_Dd2trunc is missing this residue and

is most likely mislocalised in *P. knowlesi.* This could not be confirmed by immunofluorescence assay (IFA) as the only available antibodies for PfCRT (rabbit polyclonal, obtained from MR4) react with an epitope in the C-terminus of PfCRT (56) which is missing in the truncated protein.

The same anti-PfCRT polyclonal was used to evaluate the localisation of full-length PfCRT in *P. knowlesi* pDC_PfCRT_HB3 and pDC_PfCRT_Dd2 transfectants by IFA. However the antibody generated a lot of background noise and the location of PfCRT could not be confirmed.

Since the correct localisation of PfCRT in *P. knowlesi* could not be verified by IFA, the location of a GFP-tagged PfCRT fusion was visualised by confocal microscopy. The fusion of *pfcrt* and *gfp* was generated by PCR and cloned into the expression vector PkCRT-GFP to replace the *pkcrt-gfp* fusion. This expression vector was transfected into *P. knowlesi* using the Amaxa transfection system. Parasites emerged under pyrimethamine selection within ten days. *P. knowlesi* parasites expressing PfCRT-GFP exhibit GFP fluorescence with a similar localisation pattern to parasites expressing PkCRT-GFP (Fig. 16), in vacuoles also containing haemozoin pigment confirming that full-length PfCRT localises correctly to *P. knowlesi* food vacuoles.

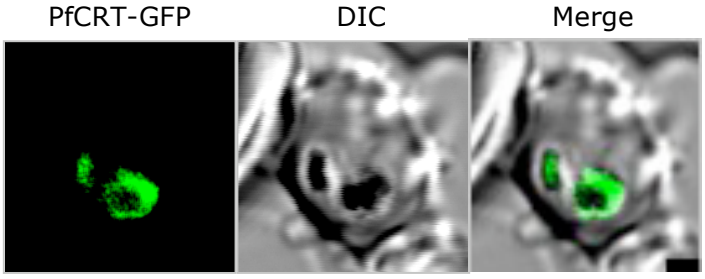

Figure 16. Confocal imaging of full length PfCRT-GFP in *P. knowlesi*. The fluorescently tagged protein can be seen colocalising with haemozoin, confirming its correct targeting to food vacuoles. Scale bar 1μm.

4.3.4. Episomal expression of full-length PfCRT Dd2 in *P. knowlesi*.

To express the full-length PfCRT Dd2 allele in *P. knowlesi*, the truncated open reading frame of PfCRT in pDC_PfCRT_Dd2trunc was replaced with the full-length Dd2 open reading frame to generate the pDC_PfCRT_Dd2full expression episome, which was transfected into *P. knowlesi* using the Amaxa transfection system. Parasites emerged under pyrimethamine selection within ten days and a drug assay was carried out (Fig. 17). This preliminary data shows that parasites expressing the full length Dd2 allele demonstrate a 4.3-fold increase in IC_{50} (Table 2) to chloroquine compared with wild-type *P. knowlesi* parasites or *P. knowlesi* parasites overexpressing the CQS allele.

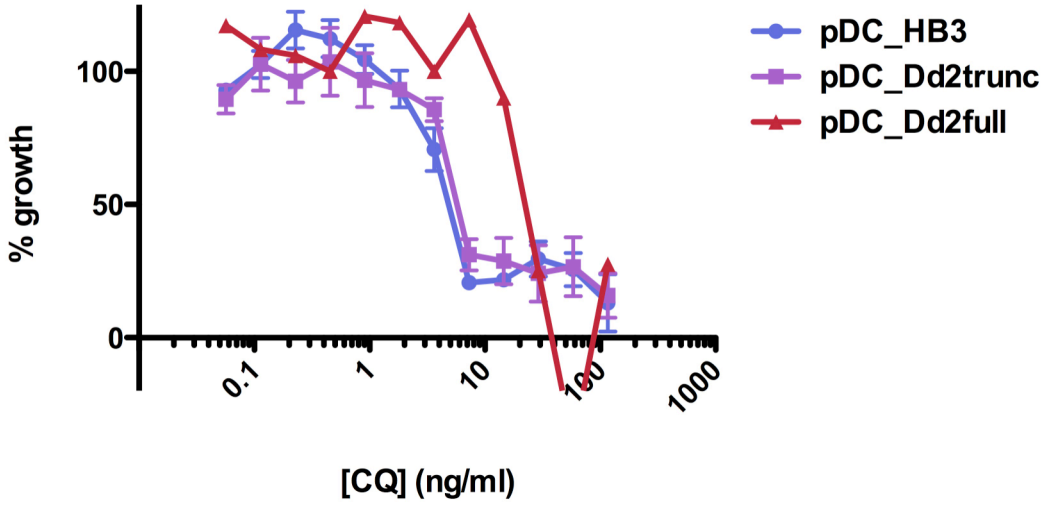

Figure 17. Comparison of growth inhibition curves of *P. knowlesi* transfectants expressing PfCRT alleles from pDC episome. Curves for pDC_HB3 (blue) and pDC_Dd2trunc (pink) represent the mean and standard error from three independent assays. Inhibition curve (mean from triplicate wells in a single assay) of parasites overexpressing the full-length PfCRT Dd2 allele (red) indicates increased tolerance to chloroquine.

Table 2. Chloroquine IC_{50} values of *Plasmodium* parasites overexpressing *pfcrt* alleles *in trans*. *Fidock *et al*.(56)

Parasite	**Episome**	**IC_{50} (nM)**
P knowlesi	-	16.14 ± 1.07
	pDC_PfCRT_HB3-FLAG	14.80 ± 1.79
	pDC_PfCRT_Dd2trunc	17.90 ± 0.95
	pDC_PfCRT_Dd2full	69.1
P. falciparum GC03*	-	31 ± 3
	pDC_PfCRT_HB3-FLAG	32 ± 2
	pDC_PfCRT_Dd2trunc	53 ± 11
P. falciparum Dd2*		278 ± 27

5. Discussion

5.1. *P. knowlesi* food vacuole morphology.

The differences in food vacuole morphology between *Plasmodia* species was first described in a number of early electron microscopy studies in which different human, primate, rodent and avian *Plasmodium* species were examined (3, 141). These studies indicated some differences between the species but more extensive investigation into the biogenesis of the food vacuoles was not possible due to the limited techniques available. Interest in the haemoglobin uptake pathway of *P. falciparum* has emerged with the publication of a number of independent studies in the past few years. These have focused solely on *P. falciparum* and while using much more modern and informative techniques, appear to have largely ignored the existing body of electron microscopy work in different *Plasmodium* species. Some of the uptake mechanisms proposed such as vesicle-independent transport of haemoglobin to the food vacuole (94) and a large phagotropic event in the ring stage (53) contradict the model described by Slomianny (162). However the remaining descriptions of *P. falciparum* haemoglobin uptake are largely consistent with Slomianny's description of micropinocytosis occurring in ring stages, taken over by cytostomal uptake as the parasites mature to trophozoites (1, 44, 165). *P. falciparum* and the avian parasite *P. lophurae* were earlier identified as displaying different food vacuole formation compared to the other *Plasmodium* species, where vesicles derived from micropinocytosis and the cytostome were combined very early in the asexual cycle to form a single central food vacuole to which all the smaller vacuoles are trafficked to (141). Considering that there are differences between the species, the study of haemoglobin uptake should include non-falciparum *Plasmodium* species such as *P. vivax* and *P. knowlesi*. This knowledge will be applied to the development of antimalarial therapies which are effective against all *Plasmodium* species which infect man.

In this study, a number of specific features of *P. knowlesi* food vacuole biology were investigated using molecular biology and imaging techniques. The distribution of haemozoin, the presence of acidic compartments and the location of known food vacuole proteins were examined to compare the food vacuole dynamics of *P. knowlesi* to the relatively well-characterised *P. falciparum* food vacuole.

Differences in haemozoin distribution could be clearly seen in Giemsa-stained parasites, consistent with early reports that haemoglobin degration occurred in multiple locations in *P. knowlesi* and the related species (141). This was supported by LysoSensor Blue staining of live *P. knowlesi* parasites. Multiple acidic vacuoles could be seen in trophozoites and early schizonts of *P. knowlesi* and the compartments stained with LysoSensor Blue were even more numerous in *P. cynomolgi*.

Are all the different acidic compartments in *P. knowlesi* and *P. cynomolgi* functioning food vacuoles? A small number of acidic compartments were observed which did not contain visible haemozoin crystals. These are possibly food vacuoles which have been recently formed and haemoglobin degradation has not yet proceeded to the point of haemozoin formation. Compartments containing haemozoin pigment which were not acidic were also observed in a small number of schizonts. These could be food vacuoles in which the pH gradient has been lost due to laser illumination (192).

The food vacuole marker proteins PkCRT and PkPM4 were tagged with the fluorescent proteins GFP and mCherry respectively, to establish the nature of the numerous acidic vesicles and to allow imaging of live *P. knowlesi* food vacuoles without the issues of light-induced loss of pH gradient when viewing LysoSensor Blue-stained parasites.

PkCRT-GFP could be seen in most parasites throughout the cycle, but the strongest signal was seen predominantly in schizonts where the fluorescently tagged protein displayed colocalisation with pigment-containing vacuoles and LysoSensor Blue. Since the signal was strongest

at a later stage in the cycle when the majority of food vacuoles had already fused, PkCRT-GFP localisation in *P. knowlesi* parasites did not provide any useful information regarding food vacuole biogenesis or the origin of different acidic vacuoles.

PkPM4-mCherry was a much more useful marker for food vacuole dynamics as it displayed a stronger signal than PkCRT-GFP in trophozoites and early schizonts when haemoglobin uptake is at its maximum. PkPM4-mCherry could be seen in many vacuoles, both with and without pigment. Any PkPM4-mCherry vesicles which did not appear to contain pigment were most likely food vacuoles in which haemoglobin degradation had not proceeded to produce enough visible haemozoin. A faint, diffuse fluorescent signal from PkPM4-mCherry could also be seen in the daughter merozoites of segmented schizonts. Although not apically located, PkPM4 may also play a role in parasite invasion of the red blood cell. PM4 has been observed in the micronemes of the ookinete of the avian parasite *P. gallinaceum* where it is involved in invasion of the mosquito midgut (98). A role for PgPM4 in merozoite invasion of the red cell has not yet been described, but could explain the presence of PkPM4 on the surface of the merozoites. Alternatively, the protein may be localised to the merozoite surface in preparation for haemoglobin degradation in the next cycle.

It was considered that the compartments visible by LysoSensor Blue staining and fluorescent protein visualisation might be interconnected as part of a single tubular network derived from the cytostome, similarly to the uptake mechanism proposed by Slomianny and Elliott (53, 162). This could be established by FRAP (fluorescence recovery after photobleaching) microscopy to selectively bleach one vesicle expressing fluorescent food vacuole protein with a laser, then monitor if the fluorescent signal returns to the same spot, indicating that the vesicles are interconnected and that the fluorescent protein can redistribute to the bleached area. Unfortunately this could not be carried out on live *P.*

knowlesi parasites, as the vesicles were too small and moved within the cell too quickly. The same reasons prevented pH comparisons between different vesicles using the *P. knowlesi* parasites expressing PfPM4-pHluorin, which would also have been informative regarding the interconnectivity of the different vesicles.

Electron microscopy of *P. knowlesi* trophozoites revealed that the vacuoles form independently of each other at different points along the parasite plasma membrane and are not connected in a network. It could not be determined from these images if the vacuoles are derived from the cytostome or by micropinocytosis. Serial sectioning of embedded cells would provide more information on the uptake processes occurring in individual cells.

Transfectant parasites expressing the fluorescently-tagged food vacuole proteins were stained with LysoSensor Blue in order to further evaluate the nature of the different compartments which may be involved in haemoglobin uptake/degradation in *P. knowlesi*. These experiments demonstrated good association between both food vacuole markers and LysoSensor Blue staining. However there appeared to be a number of acidic vacuoles in *P. knowlesi* which were stained with LysoSensor Blue, but did not contain any food vacuole protein, particularly distinct in PkPM4-mCherry parasites. It is possible in confocal images that the signal for the LysoSensor Blue or the fluorescent protein are just out of the focal plane and are therefore not detected. In the case of each 2D image, a number of focal planes through the cell were checked to ensure that the presence of fluorescent signal was not missed. To confirm this, confocal microscopy was used to generate 3D stacks to compare the localisation of acidic vacuoles and PkPM4-mCherry vacuoles throughout whole parasites. These experiments clearly indicated the presence of acidic vacuoles in trophozoites and early schizonts which did not contain PkPM4-mCherry, suggesting that they may not be involved in haemoglobin degradation. These could be acidocalcisomes, acidic storage compartments for

phosphorus and cations which have been observed in *P. falciparum* merozoites (142). They may only be visible in trophozoites and early schizonts and disappear as the parasite matures because they are divided and distributed amongst the daughter merozoites and then are simply too small to be visible. If these organelles are acidocalcisomes, it is not clear why they are not also visible in LysoSensor Blue stained *P. falciparum* trophozoites (192). Since haemoglobin uptake and food vacuole formation occurs at a number of different locations on the *P. knowlesi* plasma membrane, the acidic vesicles, which do not contain haemozoin or food vacuole protein, may be haemoglobin-containing vacuoles, which simply do not have the full complement of food vacuole proteins. Food vacuole proteins such as PkPM4 may not be distributed evenly over the parasite surface and if haemoglobin uptake is occurring predominantly by micropinocytosis at multiple points all along the parasite plasma membrane, some food vacuoles may be formed lacking certain components. In *P. falciparum,* the bulk of haemoglobin uptake occurs through the cytostome(s) meaning there are fixed points where food vacuole proteins must localise before formation of a transport vesicle and transformation into a food vacuole. Due to the redundancy in the haemoglobin degradation pathway, these vacuoles may still have the capability to degrade haemoglobin although less efficiently. Alternatively, since the vacuoles ultimately fuse together, the vacuole may simply act as a storage vesicle for haemoglobin until it can fuse with another vacuole which contains the required protein.

The observations described in this study on *P. knowlesi* food vacuole morphology suggest that the bulk of haemoglobin degradation is carried out in small food vacuoles and that the processes which fuse food vacuoles in the parasite, are not active as early on in the cycle compared to *P. falciparum*. The differences in food vacuole fusion in *P. falciparum* must be further studied and compared to *P. knowlesi* to understand the molecular mechanisms of vesicle transport and fusion, with particular

focus on the role of actin dynamics in both. Micropinocytosis and cytostomal uptake may have differential dependencies on actin dynamics (53) and actin is probably involved in the subsequent trafficking and fusion of food vacuoles (53, 94, 165). The role of actin in *P. knowlesi* haemoglobin uptake and food vacuole fusion should be investigated. If the parasite displays a preference for micropinocytosis and does not require food vacuole fusion to degrade haemoglobin, does this mean that it will be less susceptible to actin inhibitors? It would also be interesting to evaluate the factors involved in vesicle trafficking and fusion. In *P. falciparum* there appears to be a strict trafficking pathway for vacuoles directly to the main food vacuole. Do food vacuoles in *P. knowlesi* follow a pre-defined transport pathway or do they move randomly within the cell until coming into contact with each other and fusing, eventually forming a single vacuole in schizont? These vacuoles containing haemozoin can be seen moving very fast within the parasite cell when viewed under the light microscope but this movement does not appear to be purposeful. This stochastic movement could be due to Brownian movement or may be an artifact of illumination.

The host cell may have an important influence on the uptake of haemoglobin. A single strain of *P. berghei* (N strain) displays two different patterns of haemoglobin uptake depending on whether the parasite has invaded a reticulocyte or a mature erythrocyte (163, 164). *P. berghei* parasites in mature red blood cells display an extensive tubular network derived from the cytostome, from which food vacuoles bud. Parasites of the same strain in reticulocytes however did not form a tubular network however and appeared to ingest haemoglobin primarily by micropinocytosis (163, 164). This could explain why the vesicles containing pigment in *P. vivax* and *P. cynomolgi* are much smaller than in *P. falciparum* or *P. knowlesi* as the bulk of haemoglobin uptake is mediated through pinocytosis at a number of locations over the cell surface, with the ingestion of much smaller portions of haemoglobin. It

would be interesting to study the food vacuole morphology of *P. falciparum* and *P. knowlesi* in both reticulocytes and mature red blood cells to determine if these parasites, which can invade both types of cells, display alternate uptake mechanisms depending on the host cell.

The experiments here indicate that the formation of food vacuoles differs considerably between species of *Plasmodium* that infect man. This should be considered in subsequent studies on food vacuole biogenesis and efforts should be made to understand the differences in this process between species.

To explore *P. knowlesi* food vacuole morphology in more depth, the respective roles of the two food vacuole marker proteins, PkPM4 and PfCRT were further investigated.

5.2 Knock-down of PkPM4 in *P. knowlesi*.

The plasmepsins are *Plasmodium* aspartic proteases, four of which are located in the *P. falciparum* food vacuole and are involved in the degradation of haemoglobin. All four have been knocked out in *P. falciparum* and the parasites remain viable, although they display a slight growth deficiency, particularly when the parasites are grown in medium with no free amino acids (16). While the plasmepsins are not essential in *P. falciparum*, there remains considerable interest in them as potential drug targets since inhibition of the plasmepsins impedes growth of *P. falciparum* (59, 104, 137).
The knock-out of *pbpm4* in the rodent malaria *P. berghei* yielded viable parasites, which grew to a very high parasitaemia in mice. However, the knock-out parasites did not follow the normal course of infection. The mice did not develop cerebral malaria, could clear the parasites entirely and were protected from subsequent challenge for at least one year (166).

These studies establish the food vacuole plasmepsins as interesting candidates for study for the development of antimalarials in addition to the potential for the development of live-attenuated vaccines. But the systems which were used are limited to either *in vivo* or *in vitro* study and the *in vivo* data has been generated in a rodent model which may differ in the course of malaria infection and the host response in humans. *P. knowlesi* is closely related to a relevant human pathogen, *P. vivax* (123) and is becoming recognised as a serious human zoonosis in its own right (36). It can be used for *in vitro* manipulations as well as *in vivo* studies in an experimental host which is phylogenetically closely related to humans, yielding biologically relevant data. This identifies *P. knowlesi* as a versatile and informative system with which to study the potential of parasite proteins as drug targets or live-attenuated parasites as vaccines. Thus,

the essentiality of the single food vacuole plasmepsin, PkPM4 in *P. knowlesi* was investigated initially in *in vitro* studies.

The disruption of the *pkpm4* by double crossover integration could not be achieved with three different knock-out constructs, suggesting that the gene was essential. However, recent experiments in our lab suggest that double crossover integration may not be very efficient using the Amaxa nucleofector system as compared to the BioRad system. Therefore, no absolute conclusions can be made on the essentiality of *pkpm4* based solely on the knock-out attempts. To further probe the essentiality of *pkpm4* and to study the phenotype of parasites in which the PkPM4 protein is absent, the regulatable FKBP destabilisation domain (DD) system was applied for the first time in *P. knowlesi*. The DD tag allows for control of the levels of protein in response to the concentration of the compound Shield-1. In the absence of Shield-1, the protein is degraded, on addition of Shield-1 to the culture medium, the Shield-1 binds to the DD tag and prevents degradation of the protein. The *pkpm4* locus was modified by double crossover integration (using the BioRad system) to generate the full *pkpm4* ORF with an in-frame 3' *dd* tag under the endogenous *pkpm4* promoter, generating parasites pm4DD.

To confirm control of PkPM4DD in response to Shield-1 concentration, Western blots were carried out with two different antibodies directed against the DD tag, but detection of the PkPM4DD protein was unsuccessful. A polyclonal antibody specific for PkPM4 is currently in production and will soon be available for the comparison of PkPM4DD levels in +Shield-1 and –Shield-1 cultured parasites.

Although control of PkPM4DD levels in response to Shield-1 has not yet been confirmed, pm4DD parasites display a distinct phenotype in the absence of Shield-1, highly suggestive of regulated protein levels. In amino acid-limited medium, the morphology of Giemsa-stained pm4DD

parasites is different compared to wild-type parasites in the absence of Shield-1. The pm4DD parasites do not grow within the red blood cell to the same size as the wild-type parasites (Fig. 13O, P). This is probably a consequence of inhibition of protein synthesis, due to the decreased availability of amino acids in amino acid-limited medium and insufficient levels of a functional PkPM4. The appearance of pm4DD parasites in amino acid-limited medium (particularly in the absence of Shield-1) was problematic for accurate evaluation of the growth rates of the cultures. A large proportion of pm4DD parasites appeared to be dead in the Giemsa smears, introducing a level of variation in the parasite counts. This may be improved by using FACS analysis of fluorescently stained parasites differentiating dead and living parasites. This could then be incorporated into the calculations of parasitaemia. In spite of this complicating factor, the parasite levels in pkpm4DD cultures were suitably quantified to allow comparison of pm4DD growth rates. Pm4DD parasites display a significant difference in growth rate in response to Shield-1 in both complete and amino acid-limited media (Fig. 14B, C, E, F), suggesting Shield-1-dependent control of PkPM4DD levels. The growth of wild type *P. knowlesi* parasites remains unchanged in the absence of free amino acids in the medium, indicating that the haemoglobin degradation pathway provides sufficient amino acids for protein synthesis. The wild type parasite is also unaffected by the presence of 1μM Shield-1. However the growth of pm4DD parasites in complete medium is lower compared to the wild-type, despite the presence of Shield-1 which should stabilise the PkPM4DD protein. There may be several reasons for this. The Shield-1 compound may not stabilise sufficient levels of the enzyme to efficiently process enough haemoglobin to provide sufficient amino acids for protein synthesis. Increasing the Shield-1 concentration to 3μM did not cause pm4DD growth to revert to wild type levels (data not shown), indicating that Shield-1 concentration may not be the limiting factor. Alternatively, the presence of the DD tag on PkPM4 may interfere with the trafficking of the protein to the food vacuole, or inhibit the function of the protein.

Another reason for lower growth rates of pm4DD in the presence of Shield-1, could be due to modification of the *pkpm4* locus i.e. changing the endogenous *pkpm4* 3'UTR to the *pycrt* 3'UTR which could alter *pkpm4* transcription levels. This could be verified by quantitative PCR, however our primary interest is in the control of PkPM4DD protein levels in response to Shield-1 and this data will be available soon.

The presence of Shield-1 had a significant effect on pm4DD parasites in both complete and amino acid-limiting medium, however the effect of Shield-1 was more significant on pm4DD parasite growth in amino acid-limited medium (Fig. 14E, F). This is an indication of the importance of PkPM4 for efficient haemoglobin degradation, particularly when external amino acid levels are low. However, *P. knowlesi* pm4DD parasites grown in the absence of Shield-1, demonstrate slightly higher growth rates than the *P. falciparum* quadruple knock-out parasites in amino acid-limiting medium (16). This could be due to a number of reasons, such as "leakiness" of the DD-tag (7) in which a small level of PkPM4DD may escape degradation and is active in the food vacuole. In addition, the use of rhesus serum to supplement the medium, instead of AlbumaxII, might mean there are more free amino acids available to the parasites, although these levels are clearly much lower than amino acid levels in complete culture medium. These data suggest that PkPM4DD levels are regulated by Shield-1, but this is awaiting confirmation by Western blotting.

Is PkPM4 essential for *in vitro* growth of *P. knowlesi*? Knock-out experiments suggest that it is, since the *pkpm4* ORF was refractory to disruption, however this may be due to a technical issue rather than the essentiality of *pkpm4*. This inducible knock-down experiments using the DD-tagged PkPM4 indicate that *P. knowlesi* is viable without (or with low levels of) a functional PkPM4 in the food vacuole, but growth rates are diminished. Quantification of PkPM4DD levels in +Shield-1 and –Shield-1 parasites is crucial before any conclusions about the essentiality of PkPM4

may be made, however the appearance and growth rate of PkPM4DD parasites in amino acid-limiting medium in the absence of Shield-1 strongly suggest that the insufficient levels of a functional PkPM4 enzyme has an adverse effect on parasite viability and this may explain why the knock-out parasites are very difficult to obtain. In addition, these experiments underline the value of appropriate regulatable systems in *Plasmodium*.

The cysteine protease orthologues of the *P. falciparum* falcipains are also present in the food vacuole and probably compensate for the absence of the plasmepsin. In this case, haemoglobin degradation is less efficient but can still proceed at a rate which provides sufficient amino acids to support parasite growth. It may also be possible that there are additional proteases present in the *P. knowlesi* food vacuole. *P. falciparum* has created redundancy in this catabolic pathway through duplication of the plasmepsins, perhaps the other *Plasmodium* species that all have a single plasmepsin in their genomes, have evolved an alternative mechanism of redundancy in the form of other proteases which have yet to be identified in the food vacuole.

Although the knock-down of PkPM4 is clearly detrimental to *P. knowlesi* erythrocytic growth when there are no available amino acids in the medium, this may not be an accurate representation of *in vivo* conditions in the host. It would be interesting to evaluate the viability and virulence of the *P. knowlesi* pm4DD knock-down parasites in the experimental primate host, to compare the course of infection with the *P. berghei* plasmepsin knock-out in mice. Given the remarkable observation of long-lasting protective immunity induced by the plasmepsin knock-out *P. berghei* in mice, it would be interesting to investigate if the *P. knowlesi* plasmepsin knock-down parasites can induce a similar protective response in primates, then opening avenues to the development of live-attenuated vaccines.

The levels of redundancy in this *Plasmodium* biochemical pathway reflects the importance of this pathway to the parasite, but will certainly blight any drug strategies which may be developed against any single component of the pathway such as the plasmepsins. For successful inhibition of parasite growth by disruption of the haemoglobin degradation pathway, multiple enzymes will have to be targeted. However, the potential of genetically modified parasites as live-attentuated vaccines remains intriguing and the *P. knowlesi* pm4DD parasites could be a useful tool to gain valuable insight into this promising route to an effective malaria vaccine.

5.3. Heterologous expression of PfCRT in *P. knowlesi.*

Chloroquine resistance in *P. falciparum* has been well characterised with PfCRT convincingly implicated as the main facilitator of chloroquine transport out of the food vacuole. The second most prevalent human malaria species, *P. vivax* has also developed resistance to chloroquine (40 years behind *P. falciparum*) but there has been no factor definitively linked to chloroquine treatment failure. PvCRT alleles in *P. vivax* chloroquine resistant strains have not been found to carry any mutations compared to CQS strains. This indicates that a different mechanism of resistance is present in *P. vivax*. In addition, a recent study on *P. vivax* clinical isolates reports that *P. vivax* rings are more susceptible to chloroquine than trophozoites (154), unlike *P. falciparum,* where trophozoites are the most susceptibile stage to chloroquine. This is suggestive of a different mechanism of action of the drug between the species. Given the difficulties associated with *in vitro* cultivation of *P. vivax*, the closely related *P. knowlesi* parasite was used to investigate the ability of PfCRT to modulate CQR. If the expression of a chloroquine resistant allele of PfCRT in the *P. knowlesi* food vacuole modulates the chloroquine sensitivity of *P. knowlesi*, it would implicate the food vacuole as a site of action for chloroquine in "vivax-type" parasites.

The endogenous *pkcrt* could not be replaced with either CQS or CQR alleles of *pfcrt*. This may be due to the essential nature of the gene, where any modification of the *pkcrt* locus would affect parasite viability It may also be due to the unavailability of the locus for double crossover integration. This can be tested by attempting heterologous integration at the *pkcrt* locus, in which an integration construct replaces the genomic *pkcrt* with a cDNA copy of itself. This was attempted once with the Amaxa transfection system without success. In the course of this work however, doubts were raised as to the efficiency of the Amaxa system compared to the Biorad system for double crossover integration in *P. knowlesi*, thus

the difficulties encountered in replacing *pkcrt* with *pfcrt* may also simply be explained by technical issues.

As an alternative to replacement of *pkcrt*, episomal overexpression of *pfcrt* should give an indication if chloroquine resistant alleles of *pfcrt* would be capable of modulating chloroquine resistance in *P. knowlesi*. Overexpression of the HB3 (chloroquine sensitive) allele of *pfcrt* did not modulate the sensitivity of the parasites to chloroquine compared to wild-type *P. knowlesi*. This was to be expected since there are no mutations present in the protein which would allow it to facilitate chloroquine diffusion/transport out of the food vacuole, and overexpression would not change this.

The overexpression of Dd2 allele of PfCRTtrunc (missing ten amino acids from the C-terminus) gave rise to a 1.5-fold increase in CQ IC_{50} in *P. falciparum* (56) but did not significantly affect the sensitivity of *P. knowlesi* to chloroquine. This can be explained in light of the recent study published by Kuhn *et al*. in which they demonstrated that the threonine residue at position 416 (missing in PfCRT Dd2trunc) was required for correct targeting of PfCRT to the *P. falciparum* food vacuole (90). A truncated PfCRT molecule which was missing this residue was localised to the *P. falciparum* plasma membrane. So it is possible that PfCRT Dd2trunc did not modulate the tolerance of *P. knowlesi* to chloroquine as it may have been mistargeted to the parasite plasma membrane. It is intruiging that the truncated PfCRT Dd2 molecule increased the chloroquine IC_{50} of the chloroquine sensitive GC03 strain *P. falciparum* 1.5-fold, considering that the protein did not have the necessary sequence information for correct localisation to the food vacuole. Since the truncated PfCRT is trafficked as far as the plasma membrane, it is possible that a small proportion of truncated PfCRT was located close to the cytostome or at other points of haemoglobin uptake on the membrane and by chance was transported to the food vacuole in the haemoglobin transport vesicles along with other food vacuole proteins which are trafficked in this

manner. A similar, 1.5-fold increase in IC_{50} was not displayed when the truncated PfCRT was overexpressed in *P. knowlesi*. *P. knowlesi* has never been under any form of selection pressure from chloroquine, while even the CQS *in vitro* strains of *P. falciparum* have been adapted after the introduction of chloroquine to the clinic, so it can be assumed that they have accumulated some level of background mutations which can modulate chloroquine tolerance. It has recently been reported that the genetic background of *P. falciparum* parasites is important in "fine-tuning" the level of chloroquine tolerance in the presence of mutated *pfcrt*. Different chloroquine sensitive strains of *P. falciparum* were transfected to replace the endogenous *pfcrt* with a chloroquine resistant *pfcrt* allele (from *P. falciparum* 7G8). The different strains all displayed different levels of chloroquine tolerance despite expressing the same *pfcrt* allele (179). It is relevant to note that the GC03 strain in which the episomal expression of truncated PfCRT increased the IC_{50} 1.5-fold (56), displayed the largest increase in chloroquine IC_{50} of all the chloroquine sensitive lines in which the *pfcrt* allele had been replaced (179). This indicates that GC03 has probably accumulated most of the additional polymorphisms in other loci which contribute to the chloroquine resistance phenotype and therefore can be expected to display the most significant change in chloroquine tolerance, even with a small quantity of PfCRT Dd2trunc located in the food vacuole.

The full-length Dd2 PfCRT allele could not be overexpressed in *P. falciparum* from a plasmid (56), however the full-length Dd2 PfCRT allele was successfully overexpressed in *P. knowlesi*, although the parasites displayed a slight growth deficit. Preliminary data (from a single assay in triplicate wells) on these transfectant parasites, indicates that the full-length PfCRT is capable of modulating chloroquine tolerance of *P. knowlesi*. This is the first evidence of the modulation of chloroquine resistance by PfCRT in a non-falciparum *Plasmodium* parasite.

The four-fold increase in IC_{50} conferred by overexpression of PfCRT Dd2full in *P. knowlesi* does not exceed the threshold for chloroquine resistance in the clinic (80-100nM) (52). It is comparable however, to the increase in chloroquine IC_{50} of the chloroquine sensitive *P. falciparum* strain 3D7, when the endogenous *pfcrt* was replaced with the chloroquine resistant 7G8 *pfcrt* allele (179), emphasising the importance of mutations in other loci to the chloroquine resistance phenotype. It must also be kept in mind that episomal expression of a gene, even under a strong constitutive promoter, can result in varied expression levels between parasites, due to differences in episome copy number. Therefore a drug assay on episomal transfectants is assaying a heterogenous population of parasites with differing levels of gene expression. For a more conclusive evaluation of the role of PfCRT in modulating chloroquine tolerance in *P. knowlesi*, *pfcrt* should be expressed in *P. knowlesi* without expression of the endogenous *pkcrt*. This study demonstrates that direct replacement of *pkcrt* with *pfcrt* is challenging. However, an integration construct could be engineered to replace *pkcrt* with a copy tagged with the FKBP destabilisation domain, allowing control of PkCRT levels. The same integration construct could carry a *pfcrt* expression cassette, which would generate in a parasite line which bears both PkCRT and PfCRT in the presence of Shield, but only PfCRT when Shield is absent, thus allowing evaluation of the chloroquine sensitivity of the parasite due entirely to the PfCRT molecule.

This study has demonstrated that chloroquine must exert a toxic effect in the food vacuole of *P. knowlesi*, a "vivax-type" parasite, as a molecule located in the food vacuole modulates the activity of chloroquine in the parasite. However, there may be an additional site of action for chloroquine in *Plasmodium* parasites. In consideration of the reported difference in chloroquine stage sensitivity between *P. falciparum* and *P. vivax* (154), it is possible that the toxic effect exerted by chloroquine is more potent in different locations in different species. A small number of

studies on *P. falciparum* suggest that the nucleus may be an additional site of chloroquine action. Chloroquine is known to intercalate with double stranded DNA (32, 124) and chloroquine treatment has been shown to cause DNA fragmentation in a chloroquine sensitive strain *P. falciparum* but not a chloroquine resistant strain (128). It has been reported that a *P. falciparum* chloroquine resistance marker protein with polymorphisms which correlate with choroquine resistance, localises to the nucleus in chloroquine sensitive parasites but can be found in the cytoplasm around the nucleus in chloroquine resistant parasites (99, 100). Chloroquine sensitive *P. falciparum* schizonts have also been reported to produce less nuclei after chloroquine treatment, possibly due to interference of chloroquine with DNA synthesis and the merozoites which are formed after chloroquine treatment display a "delayed death" in the next cycle (63). If the nucleus of *P. vivax* is more susceptibile to chloroquine toxicity than *P. falciparum*, it may explain the differential stage sensitivities and possibly the difference in resistance mechanisms between the two parasite species.

Chloroquine resistance in *Plasmodium* appears to be a multifactorial response of the parasite to which, PfCRT is most strongly associated to, but by no means the sole contributor. It would be interesting to further investigate other proteins which contain polymorphisms correlating to chloroquine resistance in order to identify players in *P. vivax* drug resistance and improve our understanding of the mode of action of chloroquine in all *Plasmodium* species.

6. Conclusions.

A number of facets of food vacuole biology of *P. knowlesi* have been described and compared to existing data on *P. falciparum* food vacuole biology. The morphology of this lysosome-like organelle is strikingly different between *P. falciparum* and *P. knowlesi* and the other "vivax-type" parasites examined. It is unclear if this stems from differences in uptake mechanism or vesicle fusion dynamics. These differences should be borne in mind if inhibitors of these essential processes are to be developed as antimalarial compounds.

The implementation of an inducible protein expression system in *P. knowlesi* for the first time allowed the characterisation of the phenotype of *P. knowlesi* lacking a functional plasmepsin protein. Knock-down of PkPM4 in *P. knowlesi* resulted in a growth deficit in both amino acid-rich and amino-acid limited media indicating a disruption in nutrient acquisition. If complete knock-down of PkPM4 can be confirmed, this parasite line may be used for *in vivo* studies to assess the potential of live-attenuated *Plasmodium* parasites as a malaria vaccine.

Episomal expression of a PfCRT allele from a chloroquine resistant *P. falciparum* parasite in *P. knowlesi* demonstrated that PfCRT is sufficient to modulate the chloroquine sensitivity of *P. knowlesi*. This indicates that chloroquine does exert a toxic effect in the food vacuole of "vivax-type" parasites despite apparent differences in stage sensitivity and a different mechanism of choroquine resistance in *P. vivax* compared to *P. falciparum*.

Existing data on *Plasmodium* food vacuoles is limited to observations in *P. falciparum*, which fails to identify any species-specific differences. *P. falciparum* can only be studied *in vitro*, apart from highly artificial *in vivo* infections in splenectomised *Aotus* monkeys or humanised mice. This

limits *in vivo* studies to artificial conditions which do not necessarily represent natural infection or rodent models which may not mirror human disease. The studies described in this thesis highlight the value of *P. knowlesi in vitro* culture and transfection technology for understanding the biology of *Plasmodium* parasites which infect humans, considering the intractability of *P. vivax*. *P. knowlesi* culture and transfection can be effectively used in parallel with *P. falciparum*, to complement experiments in *P. falciparum* and give a more broad and informative view to the biology of more *Plasmodium* species which infect man.

7. References.

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