Understanding the mechanisms of protein export in *Plasmodium berghei* liver infection

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Abstract

The malaria parasite exports a large repertoire of proteins into the host erythrocyte during blood stage infection. This essential process of host cell remodelling is largely dependent upon the cleavage of a conserved amino acid motif, termed the Plasmodium Export Element (PEXEL). The aspartyl protease plasmepsin V is the enzyme responsible for this cleavage event and previous attempts at genetic deletion have been unsuccessful. Although years of research have revealed the mechanisms behind protein export in erythrocytic infection, less is known about how the parasite exports proteins during the liver stage of malaria infection. This dissertation identifies the molecular mechanisms the parasite requires to facilitate export into the host hepatocyte.

Using the Flp-FRT system to conditionally control the expression of plasmepsin V across the life cycle of *P. berghei* (rodent malaria), we achieved a complete deletion of the 3'UTR of the plasmepsin V gene, specifically in sporozoites. Analysis of this parasite line revealed a defect in liver stage development as the parasites arrested from 6 h following sporozoite infection in mice. Infected mice did not develop patent blood stage infections. Together this suggests an essential role for plasmepsin V during liver stage malaria infection.

In order to understand how the parasite exports proteins during liver stage infection, the importance of the PEXEL motif needed to be addressed. We identified several PEXEL-containing protein candidates and examined their export during liver stages to show that their export is dependent upon the presence of a functional PEXEL motif or plasmepsin V. These important findings may help the further identification of exported proteins.

It is ultimately important to also contextualise how parasitic infection may change the dynamics of hepatocyte homeostasis, possibly by the result of exported proteins. Recent studies have shown the parasite's ability to inhibit host hepatocyte apoptotic mechanisms during infection. We examined this using

a knockout of key apoptotic inhibitory regulators (cIAP1/2) and found that their absence in mice caused an increase in resistance to liver stage infection. Chemical anatagonism of these apoptotic regulators replicated this effect, highlighting the potential for new drug targets for liver stage malaria.

This thesis explores the underlying mechanisms behind protein export in liver stage *Plasmodium berghei* infection. The results shed light on what is required for the parasite to export proteins to the hepatocyte, for the first time, confirming the importance of plasmepsin V and the PEXEL motif. These discoveries will lead to potential new drug targets and vaccines candidates, aiding the world's fight to eradicate the debilitating disease burden of malaria.

Declaration

This is to certify that:

i. The thesis comprises only my original work towards the degree of Doctor of Philosophy except where indicated in the Preface.

ii. The acknowledgement has been made in the text to all other material used.

iii. The thesis is fewer than 100,000 words in length exclusive of table, maps, bibliographies and appendices.

Pravin Rajasekaran

Preface

Unless otherwise specified, all work described in this thesis was undertaken at the Walter and Eliza Hall Institute of Medical Research in the laboratory of Dr Justin Boddey and under the co-supervision of Prof. Alan Cowman. All experiments and methods involving animals were approved by the Walter and Eliza Hall Animal Research Ethics committee.

I have assessed my contributions for each scientific chapter of this thesis as follows:

Chapter 3: 90%

Chapter 4: 90%

Chapter 5: 50%

In chapter 3, I performed all the experiments involving the generation of parasite lines and downstream analysis with *P. berghei* infections. Analysis of LISP2 export (the counting of cells and measuring cell size) and LISP2 PEXEL processing was performed by Bethany Davey (Figure 4B, 4C, 4E). Experimental planning and assistance with methods were given by other members of the laboratory including Matthew O'Neill, Sash Lopaticki, Annie Yang and Sarah Erickson.

In chapter 4, I performed most of the experiments involving the generation of the clonal parasite lines (WT and mutant PEXEL) and analysis in blood and liver infections. Generation of the PBANKA_0700700 knockout parasite line and analysis of the 0700700-mCherry line in blood and liver stages were done in the laboratory of Dr Blandine Franke-Fayard.

In chapter 5, the work obtained and presented in this thesis was due to the combined efforts of myself, Sash Lopaticki and Dr Greg Ebert. I initiated all the *P. berghei* infections in mosquitoes and mice. Dissection and generation of RNA was done together by all three of us. Sash Lopaticki performed and generated the RT-PCR data presented in this chapter. I performed the counting of parasitemia and the days to patent infection. Whilst most of the data presented in this chapter are my contributions to the study, I have not been involved in some other aspects of this project. Once all aspects are finished, collated and prepared for publication, I will be considered one of the secondary authors to this study.

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Finally, A large thanks needs to go to my parents. Mum and Dad, thanks for the guidance and support in my life and during the PhD. It's been a tough time but I've finally made it to the end and look forward to the next venture in life.

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Chapter 1: Introduction

1. Malaria The Disease

Malaria is caused by obligate intracellular parasites of the *Plasmodium* genus in the phylum of Apicomplexa. Methods of controlling and eradicating malaria have proven to be difficult as malaria is still responsible for the deaths of 584,000 people in 2014 with over 214 million cases around the world (WHO report, 2015). Interventions to reduce malaria mortality rates and transmission have been successful in recent years, however, with the emergence of artemisinin resistant parasites (Noedl *et al.*, 2008), the need for new drugs and vaccines remains. Of the many species of *Plasmodium* that exist, there are only 5 species known to infect humans including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. *P. berghei* and *P. yoellii* are both rodent forms of malaria that have been scientifically useful in dissecting the biology of the malaria parasite.

P. falciparum causes the most deadly form of human malaria and is responsible for the morbidity and mortality of the disease in most parts of Africa. Its symptoms include fever, nausea, anaemia, shaking and muscle contractions, affecting multiple organs and systems of the body (Miller *et al.*, 2002). The most severe symptoms occur due to the decreased deformability (Dondorp *et al.*, 2000) and increased cytoadherence of infected erythrocytes to other cells and blood vessels (Ho & White, 1999). Rosette formation (erythrocyte clusters) and cytoadherence to blood vessels alter normal blood flow, causing the pathophysiological outcomes such as anaemia (Miller *et al.*, 2002). Sequestration of infected cells specifically in the brain can result in seizures, coma and further severe malaria symptoms (Idro *et al.*, 2005).

P. vivax causes less mortality than *P. falciparum*, however, it is more widespread with an estimated 2.5 billion people at risk in countries of South East Asia, South America, as well as some parts of Africa (Gething *et al.*, 2012). One of the unique features of *P. vivax* (and *P. ovale*) is its ability to develop a dormant form of infection during pre-erythrocytic stages called the hypnozoite. This form of the parasite can reside within the liver from months to years after infection, and the causes of activation and reinfection are still unknown (Chu & White, 2016). The lack of effective drugs to target the hypnozoite further complicate the eradication efforts established in countries afflicted with *P. vivax*.

The rodent malaria *Plasmodium berghei* was discovered in the host species *Grammomys surdaster* (Vincke & Lips, 1948). The biology and life cycle of *P. berghei* resembles that of the human malaria species. The application of molecular genetics to rodent malaria parasites (van Dijk *et al.*, 1996) has enabled the use of this species as an invaluable model to study parasite biology across both sexual and asexual stages of infection.

2. The *Plasmodium* Life Cycle

The injection of sporozoites from the bite of a female *Anopheles* mosquito into the skin of the mammalian host begins the pre-erythrocytic stages of the malaria parasite life cycle (reviewed in Menard et al., 2013). Sporozoites migrate through the blood stream and traverse cells (Vanderberg *et al.*, 1990) to eventually invade and manifest as a new form of the parasite within the liver of the host. The liver stage parasite is encased by a parasitophorous vacuole (formed during the invasion process from the liver cell membrane), within which it matures mitotically into a schizont containing tens of thousands of merozoites. The liver stage (or exo-erythrocytic form) of malaria is clinically silent and lasts for a period of 7-10 days in humans and approximately 2-3 days in rodents.

Despite the discovery of blood stages of malaria occurring in 1880 (Laveran, 1881), it was not until 1948 that scientists discovered the schizont forms of

Plasmodium cynomolgi in liver cells of the rhesus monkey (Shortt & Garnham, 1948). The difficulty of isolating and studying the exoerythrocytic forms has hindered our scientific understanding of this silent stage of malaria compared to the other stages of malaria infection, even to this day.

Merosomes produced in the liver stage can bud from the liver cells and enter the blood stream, rupturing and initiating the pathological asexual blood stage of malaria. Merozoites can then apically position themselves around the surface of erythrocytes beginning the process of invasion (Cowman *et al.*, 2012). The parasite generates a parasitophorous vacuole membrane during the invasion process that encases the intracellular parasite. Immediately following invasion, the parasite remodels the host erythrocyte through the export of numerous effector proteins (Boddey & Cowman, 2013). Parasites develop into ring, trophozoite and schizont stages, where the parasite can produce 16-32 daughter merozoites, which can then reinvade and continue the asexual stage of infection.

Merozoites can continue asexual replication or commit to sexual differentiation by forming gametocytes (Bruce *et al.*, 1990). The biological switch that triggers this differentiation is still unknown but involves transcriptional control mediated through lysoPC, ApiA2-g and GDV1 (Brancucci *et al.*, 2017; Filarsky *et al.*, 2018; Mancio-Silva *et al.*, 2017). During a blood meal, a female *Anopheles* mosquito can ingest mature male and female gametocytes, which then undergo fertilisation and zygote formation within the midgut. Over the next 24 hr, the parasite matures into a motile ookinete with the ability to traverse the mosquito midgut epithelium and embed itself at the basal lamina before developing into an oocyst. The oocyst undergoes sporogony, forming thousands of sporozoites within oocysts (Sinden & Strong, 1978). After maturation, sporozoites egress from oocysts and migrate to and actively invade the salivary glands, where they await injection into a new mammalian host, where they can continue the life cycle.

3. Antimalarial Vaccines and Drugs

One of the key strategies to eradicate malaria from the world involves the generation of an effective vaccine able to prevent infection in an immunised host. The complexity of the multi-host malaria parasite life cycle complicates the production of vaccines targeting various stages of parasite development. However, vaccines designed to target blood and liver stage malaria parasites have produced limited success in recent years. The vaccine FMP2.1/AS02(A), a recombinant form of apical membrane antigen 1 (AMA1) from *Plasmodium falciparum*, generated no significant protection against blood stage malaria (Thera *et al.*, 2011). Some success has been achieved with the RTSS AS01 vaccine (targeting circumsporozoite protein (CSP) in *Plasmodium falciparum*) (S Clinical Trials Partnership, 2015), however its effectiveness decreases over extended periods of time (Clinical Trials Partnership, 2014).

Injections of whole parasites, attenuated through irradiation or genetic disruption, have generated the most protection to date. Irradiating *P. berghei* sporozoites to a non-replicative state has been able to induce strong protection after injection into mice (Nussenzweig *et al.*, 1967). In more recent years, the ablation of specific liver stage genes causing parasite arrest during later liver stages has proven to be more effective than irradiation methods (Butler *et al.*, 2011). Genetically attenuated parasites (GAPs) targeting UIS3 (Mueller *et al.*, 2005), sap1 (Aly *et al.*, 2011) and p52/p36 (Annoura *et al.*, 2012) have shown promise against rodent malaria. Injection of sporozoites intravenously provides the greatest protection and generates greater CD8+ and CD4+ T-cell responses in mice (Haeberlein *et al.*, 2017). Translation of these results towards a clinical whole parasite vaccine is currently underway (Ishizuka *et al.*, 2016; Kublin *et al.*, 2017). Clinical trials involving the PfSPZ vaccine, whole parasites attenuated by irradiation, concurrent drug administration or genetic ablation, have also been partially successful, in that significant number of patients develop antibodies to CSP, however, protection wanes rapidly over time indicating that efficacy is low and the vaccine requires significant improvement before complete protection can be achieved (Olotu *et al.*, 2018; Richie *et al.*, 2015; Seder *et al.*, 2013).

Due to the lack of an effective vaccine, antimalarial drugs provide the current frontline of protection against malaria, such as artemisinin combination therapies (ACTs). ACTs are effective only against blood stage parasites, although recent interest in pharmacologically targeting liver stages of infection has emerged (Derbyshire *et al.*, 2011). Only one drug, primaquine, has shown to be useful against both liver and blood stages of infection, however, its drastic side effects in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency prevent it from being suitable for use in many cases (Baird, 2015). A newly approved liver stage therapeutic, tafenoquine, has also been tested as a prophylactic with less toxicity than primaquine (McCarthy *et al.*, 2018). Development of new liver-stage antimalarials has additional benefits, as targeting this 'bottleneck' of the parasite life cycle should delay the development of drug resistance, as liver stage infection only features one cycle of replication, giving less time and parasite numbers for drug resistance to be selected for (Mazier *et al.*, 2009). Due to the complexity of the malaria parasite life cycle and the various ways in which the parasite can remodel host cells and evade immunity, it is clear that both blood and liver stages of infection must be equally considered when targeting malaria with drugs or vaccines.

4. Molecular mechanisms of malaria pathogenesis during erythrocytic infection

4.1 Protein export and erythrocyte remodelling

During erythrocytic stages of malaria parasite infection, the parasite encompasses itself within a vacuole following invasion. In an extraordinary process of host cell remodelling, the malaria parasite exports a large repertoire of proteins across the parasitophorous vacuole membrane (PVM) and into the host cell. Proteins are trafficked via the parasite secretory pathway into the parasitophorous vacuole with an N-terminal hydrophobic signal sequence (Wickham *et al.*, 2001) and are then translocated across the vacuole and into the host cytoplasm. The result is an elaborate protein trafficking network consisting

of membranous structures, highly deformable membranes and knob-like surface protrusions on the cell surface.

The membranous structures found in the host cell during blood stage development are known as Maurer's clefts . Maurer's clefts act as a sorting station and play an important role in trafficking and sorting of other exported protein complexes to the host cell cytoplasm and membrane (reviewed in Mundwiler-Pachlatko and Beck (2013)).

Protein export is responsible for the cytoadherence of infected RBCs to other cells and blood vessel walls. This phenomenon is attributed to *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) protruding from knob structures present on the RBC membrane surface (Baruch *et al.*, 1995; Leech *et al.*, 1984). The knob structures consist of knob associated histidine rich protein (KAHRP) on the cytoplasmic side (Pologe *et al.*, 1987). The *Pf*EMP1 family of proteins features over 60 *var* genes with mono-allelic expression. The parasite's ability to switch *Pf*EMP1 *var* gene expression provides antigenic variation conferring an additional mechanism of immune evasion (Smith *et al.*, 1995). Sequestration of malaria parasites to capillaries in the brain and placenta of pregnant women causes severe malaria symptoms and mortality, suggesting a direct link between protein export and virulence.

4.2 The *Plasmodium* Export Element (PEXEL)

The discovery of a pentameric N-terminal amino acid motif (RxLxE/Q/D) required for export of many *Plasmodium* proteins initiated our understanding of the malaria parasite's exportome, a list of all predicted exported proteins. The *Plasmodium* export element (PEXEL) motif (Marti *et al.*, 2004) or Host targeting (HT) sequence (Hiller *et al.*, 2004) is located 20-30 amino acids following the signal sequence and directs the protein for export across the PVM.

The mechanism behind the PEXEL motif's role in protein export remained unknown until it was identified as a cleavage site. Cleavage occurs after the

conserved PEXEL leucine residue; the mature protein is then acetylated at the new N-terminus (Boddey *et al.*, 2009; Chang *et al.*, 2008). Plasmepsin V, an ER-resident aspartyl protease has been shown to be responsible for the proteolytic cleavage of the PEXEL motif for export (Boddey *et al.*, 2010; Russo *et al.*, 2010). The conserved arginine and leucine residues of the PEXEL are important for recognition and cleavage of the motif, whereas the 5th amino acid residue shows some redundancy and is not required for processing, although it is still important for export to the host cell. The importance of the PEXEL cleavage event was illustrated when it was found that reporter engineered to feature a mature cleaved form of a PEXEL protein (N-terminal xE/Q/D) was not exported into erythrocytes (Boddey *et al.*, 2010). A so-called "relaxed" PEXEL motif, such as RxLxxE found in RESA, can also be cleaved by plasmepsin V and is required for export, suggesting additional redundancy with the 4th and 5th residues exists (Boddey *et al.*, 2013). Several PEXEL-like peptidomimetics with high affinity for plasmepsin V have been generated and these were able to decrease levels of protein export and significantly inhibit parasite growth (Gazdik *et al.*, 2016; Hodder *et al.*, 2015; Nguyen *et al.*, 2018; Sleebs *et al.*, 2014). Furthermore, attempts to completely disrupt plasmepsin V gene expression have so far been unsuccessful, implicating plasmepsin V as a crucial enzyme for parasite survival (Boddey *et al.*, 2010; Klemba & Goldberg, 2005; Russo *et al.*, 2010).

The function of the PEXEL/HT motif and the importance of its processing for export was questioned when it was discovered that the motif reportedly binds with high affinity to the lipid phosphatidylinositol-3-phosphate (PI3P) located within the ER (Bhattacharjee *et al.*, 2012). It was shown that the RxLR targeting motif found in the oomycete *Phytophthera infestans* (Whisson et al., 2007) could also be expressed in *Plasmodium*, and target reporter proteins to the host erythrocyte (Bhattacharjee *et al.*, 2006), thus suggesting that the PEXEL and RxLR motifs function in a similar way. It was reported that binding of RxLR and PEXEL motifs to PI3P was essential for initiating protein export to the host cell and that this process occurs independent of plasmepsin V cleavage (Bhattacharjee *et al.*, 2012). Later studies revaluated the importance of plasmepsin V cleavage by showing that motifs with reported affinity to PI3P

(such as KxLxE/D/Q) were not exported to the host erthryocyte (Boddey *et al.*, 2013). It was shown that PI3P did not localise in the ER and was in fact more prevalent in the parasitic food vacuole as a result of endosomal trafficking of haemoglobin (Boddey *et al.*, 2016). Repeated experiments revealed that the PEXEL of recombinant proteins does not bind to PI3P and that plasmepsin V cleavage occurs rapidly and is essential for export (Boddey *et al.*, 2016; Sleebs *et al.*, 2014). These findings together with the inability to generate a genetic deletion of plasmepsin V suggest a critical role for the enzyme and protein export as a whole.

The PEXEL motif is highly conserved throughout all sequenced species of *Plasmodium,* however, *P. falciparum* features the largest predicted exportome (Marti *et al.*, 2004; Sargeant *et al.*, 2006), further agreeing with the idea that virulence and protein export are intrinsically linked. Plasmepsin V is also conserved across all species of *Plasmodium.* Due to the inability to culture *P. vivax* in the laboratory, little is known about the existence of export pathways in *P. vivax*. Expression of *P. vivax* PMV in *P. falciparum* enabled the analysis of the substrate specificity of the *P. vivax* enzyme, revealing an almost identical specificity to that of *P. falciparum* PMV (Sleebs *et al.*, 2014). This suggests strong conservation of the PEXEL export machinery across species of *Plasmodium*. Protein export has been identified in *P. berghei* with studies highlighting the export of PEXEL-GFP reporters (Haase *et al.*, 2013) and the presence of Maurer's cleft-like structures, IBIS (Ingmundson *et al.*, 2012). The cleavage of the PEXEL motif and the role of plasmepsin V in *P. berghei* are yet to be fully understood.

4.3 PTEX translocon

Cleavage of the PEXEL motif by plasmepsin V enables trafficking of the protein towards the parasitophorous vacuole, but an additional step in the pathway must occur to facilitate translocation across the vacuole membrane and into the host cell. A protein complex, known as the translocon of exported proteins (PTEX) (de Koning-Ward *et al.*, 2009), is responsible for the translocation. The PTEX complex comprises of EXP-2, HSP101, PTEX150, Trx-2 and PTEX88, all of which were identified by proteomic analysis of PVM and parasite membranes. An

additional accessory molecule component of PTEX, Parasitophorous Vacuolar protein 1 (PV1) was recently found to be associated with EXP-1 (Morita *et al.*, 2018). Effector proteins are required to be unfolded and then refolded following translocation via the PTEX complex (Gehde *et al.*, 2009). The PTEX proteins could potentially fit the roles required for a functional protein translocon complex, including the pore-forming nature of EXP-2 and the chaperone HSP101.

Recent studies have unravelled the link between the PTEX components and protein export by conditionally regulating expression of HSP101 and PTEX150 in *P. falciparum* and *P. berghei* respectively (Beck *et al.*, 2014; Elsworth *et al.*, 2014). Parasites were unable to grow effectively and showed a defect in protein export suggesting the essentiality for the PTEX complex and the importance for the translocation of proteins into the host cell. Cryo-electron microscopy has captured the entire PTEX complex functioning as a translocon as proteins are unfolded as they pass through the EXP-2 pore, further confirming the complexes requirement for protein export in *P. falciparum* erythrocytic stages (Ho *et al.*, 2018).

4.4 PEXEL-negative exported proteins (PNEPs)

Whilst the *Plasmodium* exportome comprises primarily PEXEL-containing proteins, there exist a number of exported proteins that do not feature the conserved motif. Analysis of the PEXEL-negative exportome reveals a large numbers of PNEPS suggesting that they form a significant part of the parasites entire exportome. Examples of PNEPS include the virulence surface antigen *Pf*EMP1, ring exported proteins 1 and 2 (Haase *et al.*, 2009), merozoite surface protein 7 related proteins (Heiber *et al.*, 2013) and skeletal binding protein 1. These PEXEL-negative exported proteins (PNEPS) are not substrates of PMV and do not contain an N-terminal hydrophobic signal sequence (Boddey *et al.*, 2013). Some PNEPS contain a transmembrane domain at the N-terminus, which is thought to act similarly to a signal sequence in that it can facilitate ER entry and downstream trafficking events (Heiber *et al.*, 2013). The export of PNEPS also relies on unfolding and translocation across the parasitophorous vacuole,

however, the translocation process is thought to be vastly different to PEXEL-mediated export (Grüring *et al.*, 2012).

4.5 Protein export in other Apicomplexans

Whilst *Plasmodium* parasites have evolved a unique protein trafficking pathway for the selection of PEXEL cargo at the ER for export to the erythrocyte (Marapana *et al.*, 2018), recent discoveries suggest PEXEL-like sequences and protein export mechanisms are conserved in other Apicomplexans (Coffey *et al.*, 2016). There is a class of effector proteins present in *Toxoplasma* that traffic through the dense granules (GRA) to the parasitophorous vacuole, the parasitophorous vacuole membrane and the host cell. One such protein, GRA16, is exported into the host cell nucleus and potentially plays a role in inhibiting host cell apoptosis and cell cycle arrest (Bougdour *et al.*, 2013). Interestingly, GRA16 also contains an N-terminal PEXEL-like motif (RRLAE). The golgi-resident Aspartyl protease 5 (ASP5), a homologue to plasmepsin V, has been identified in *Toxoplasma* and shown to cleave PEXEL-like sequences (named 'TEXEL' for Toxoplasma export element) in *Toxoplasma* (Coffey *et al.*, 2015; Hammoudi *et al.*, 2015). Genetic strategies have been successful in disrupting ASP5 expression indicating a non-essential role for TEXEL-mediated export *in vitro* (Coffey *et al.*, 2015; Hammoudi *et al.*, 2015), although parasite growth is significantly delayed and virulence in mice was significantly reduced demonstrating the ASP5 trafficking pathway is important. TgASP5 has also been showed to directly mediate the export of GRA16 to the host cell (Curt-Varesano *et al.*, 2016) and other secreted GRA proteins (GRA17 and GRA23) have been implicated in membrane pore formation (Gold *et al.*, 2015), functionally similar to that of *Plasmodium* EXP2. Additionally, Pellé et al. (2015) have reported the identification of PEXEL-like motifs in both *Cryptosporidium* and *Babesia*, providing further evidence of conservation of the export machinery within members of the Apicomplexan family.

5. Parasite-Host interactions in the liver stage of *Plasmodium* infection

5.1 Virulence mechanisms during liver infection with *Plasmodium* spp.

Whilst blood stage infection involves considerable host cell remodelling to facilitate merozoite development and evasion of host defences, the clinically silent liver stage of infection features only a single cycle of infection within the host cell. The sporozoite deposited into the host from an infected mosquito must invade a hepatocyte and progress to schizogony, where it develops into tens of thousands of liver-stage merozoites. The liver stage parasite is required to evade innate and adaptive immune pressures and scavenge nutrients from the host cell for efficient development of schizonts. These processes are likely to involve exported parasite proteins.

During the hepatocyte invasion process, in a similar manner to the erythrocytic stage of infection, the parasite concomitantly forms a parasitophorous vacuole (Lingelbach & Joiner, 1998; M. *et al.*, 1983). Studies have demonstrated that the parasite requires the uptake and exchange of host cell factors for development (Allary *et al.*, 2007; Deschermeier *et al.*, 2011; Itoe *et al.*, 2014; Meireles *et al.*, 2017). These nutrients must cross the parasitophorous vacuole suggesting that liver stage parasites employ a highly dynamic system to exchange factors with the host cell. A role for solute exchange across the PVM was recently reported for EXP2 in *P. falciparum* blood stages (Garten *et al.*, 2018) and this may also be the case in liver stages, although direct evidence has not been reported. The protein composition of the parasitophorous vacuole membrane supports this theory and further implicates parasites directly manipulating host cell processes and evading host cell immune responses (Real *et al.*, 2018; Spielmann *et al.*, 2012).

One example is destabilization of host hepatocyte p53 within *Plasmodium*-infected liver cells (Kaushansky, Ye*, et al.*, 2013). p53 is a protein able to initiate cell death processes and is important for cell cycle progression; parasitic interference with this protein and the process of apoptosis and cell cycle suggests that host-cell interaction through export could be required for liver

stage parasite development. Parasites also subvert autophagy in the hepatocyte and recruit host LC3 to the PVM via a direct interaction with the parasite protein UIS3 (Real *et al.*, 2018).

5.2 Liver stage protein export

The essentiality of protein export in blood stages has been established in *Plasmodium* through the use of molecular genetics (Maier et al Cell 2008) and development of protein export inhibitors (Gazdik *et al.*, 2016; Hodder *et al.*, 2015; Nguyen *et al.*, 2018; Sleebs *et al.*, 2014) and conditional regulation of protein export translocation machinery (Beck *et al.*, 2014; Elsworth *et al.*, 2014). Whether PEXEL mediated protein export occurs in liver stages of *Plasmodium* infection, however, is still yet to be determined. It has been shown that CSP, a protein unique in that it contains 2 PEXEL motifs, was detected in the hepatocyte cytoplasm and nucleus via immunofluorescence microscopy (Singh *et al.*, 2007). Contrary to this, another study showed using antigenic detection by MHC class II molecules that double PEXEL mutant CSP was also present in extra-parasitic compartments (Cockburn *et al.*, 2011), suggesting the export may be PEXEL independent. Furthermore, using *flp*-FRT mediated conditional gene regulation, it was found that EXP-2, the PTEX translocon component, played an important role in liver and blood stages, however, expression of the other translocon component, HSP101, could not be detected (Kalanon *et al.*, 2015). A KAHRP PEXEL-GFP reporter expressed in liver stages appeared to aggregate in the parasitophorous vacuole of liver stages and was not detected in the cytosol. This suggested that export of PEXEL proteins does not occur in liver stages. However, Orito et al. (2013) identified liver specific protein 2 (LISP2) and showed that this abundant protein is exported to the hepatocyte cytoplasm and nucleus from 36-60 h post-infection. LISP2 contains a PEXEL sequence RILAE located further downstream of the N-terminus than typically expected. It is unclear whether the export of this protein is reliant upon the same PEXEL-dependent export machinery found during erythrocytic stages of infection. Moreover, it is unknown whether plasmepsin V is required for export of other proteins during liver stage infection. Given the possibility that protein export is essential for

parasites to manipulate host cell processes needed for parasite development, a better understanding of protein export in liver stages is required.

5.3 Apoptosis of the host cell during homeostasis and infection

Apoptosis is the process in which cells undergo a form of altruistic programmed cell death for a variety of reasons, including following infection with a myriad of pathogens. During normal cell growth and development, apoptosis initiates cell turnover, ensuring the presence of healthy tissue. It can also be an important defence mechanism, being initiated by stress or damage inflicted upon the cell from cytotoxic and mutagenic sources including UV light, radiation and reactive oxygen species . Host cells can induce apoptosis in response to various infections including HIV, Hepatitis B and malaria. Unlike other forms of cell death such as necrosis, the cell death initiated by apoptosis does not cause damage to surrounding tissue (Norbury & Hickson, 2001).

Apoptotic signalling can occur in two main pathways, known as the intrinsic and extrinsic apoptotic pathways. Both pathways involve a complex signalling cascade that can induce signals from the other pathway, synergistically working together to cause cell death (Igney & Krammer, 2002).

The intrinsic pathway (also called the mitochondrial pathway) is classically associated with DNA damage or cellular stress initiating release of cytochrome c and SMAC/DIABLO from the mitochondrial membrane. Cyctochrome c can induce the formation of the apoptosome complex, leading to activation of caspase 9 and downstream activation of caspase 3 (Cohen, 1997). SMAC antagonises inhibitors of apoptosis proteins (IAP) molecules including X-chromosome linked IAP (XIAP) which is involved in inhibiting the cleavage (and hence activation) of caspase 3 by caspase 9. The presence of activated caspase 3 is a hallmark of apoptosis (Cohen, 1997).

Initiation of the extrinsic pathway of apoptosis involves the ligation of death receptors including Death receptor 5, Fas and TNFR1 (Locksley *et al.*, 2001).

Binding of Fas ligand or TRAIL to DR5 or FAS recruits the adaptor molecule FADD (Fas associated death domain protein), which can then activate caspase 8. Active caspase 8 can cleave and activate downstream caspase 3, leading to apoptosis. Additionally, caspase 8 can also cleave BID (BH3 interacting domain death agonist), which can consequently translocate to the mitochondria, stimulating release of cytochrome c and SMAC in the intrinsic pathway.

The binding of TNFα to TNFR1, in the absence of cellular inhibitor of apoptosis proteins (cIAPs), initiates the association of Rip1 with TRADD (Elmore, 2007; Hsu *et al.*, 1995; Locksley *et al.*, 2001). The recruitment of caspase 8 can then form the pro-apoptotic cytoplasmic complex, leading to activation of caspase 8, hence, activation of caspase 3 and apoptosis.

Cells must achieve a sense of balance when dealing with factors that induce cell growth and cell death; too much apoptosis can lead to tissue destruction and degenerative diseases whereas too little can lead to tumour growth and autoimmune diseases. One major family of proteins involved in the regulation of apoptosis in are the inhibitors of apoptosis proteins (IAPs) (Silke & Meier, 2013). There are a total of 8 IAPs including X-chromosome linked IAP (XIAP) and the cellular inhibitors of apoptosis 1 and 2 (cIAP1, 2). Despite their name, only XIAP has been confirmed to directly bind to caspases 3, 7 and 9 to inhibit apoptosis (Eckelman *et al.*, 2006).

cIAPs 1 and 2 are both involved in TNFR1 signalling in the canonical pathway for NfkB activation (Silke & Meier, 2013). Whilst TNFR1 signalling can act as a death receptor through RIP1-TRADD-caspase 8 interactions, cIAPs 1/2 are able to ubiquitylate RIP1, a process which also causes self-ubiquitylation (Silke & Meier, 2013). This ubiquitylation event prevents the interaction of RIP1 and TRADD. Instead of a signalling cascade leading to apoptosis, a platform is formed for the recruitment of the signalling molecules TAB, TAK1 and IKK. Further signalling events initiate the degradation of IKB and translocation of p50 into the nucleus allowing for transcription of NfKB. NfKB is typically associated with a pro-inflammatory and pro-survival signalling response. Ultimately, the

presence/absence of cIAPs can heavily influence the TNFR1 signalling pathways leading to either a pro-survival or pro-apoptotic response. The cIAPs are therefore the molecular switch in apoptotic signalling that determine fate with respect to cell survival or cell death. For this reason, the function of cIAPs during *Plasmodium* liver infection is an important, yet completely neglected, area for investigation.

5.4 Apoptosis in infectious diseases

Whilst cell growth and cell death mechanisms are typically associated with cancer, autoimmune and degenerative diseases, these signalling pathways are also very important when considering host defence during infectious diseases.

Host cell death is a common defence mechanism against pathogens. Recently this was demonstrated for the liver-tropic infection caused by Hepatitis B virus (HBV). In a mouse model of HBV, clearance of infected hepatocytes through the initiation of apoptosis was shown to occur in a TNFα-dependent manner. Both TNFα and TNFR1 are upregulated during infection and treating infected mice with TNF-neutralising antibody resulted in impaired control of HBV infection (Ebert, Preston*, et al.*, 2015b). This demonstrates that extracellular cell death signalling is an important pathway for host defence. This raises the question of whether cIAPs are involved in HBV infection. Indeed, it was subsequently shown that cIAPs were directly responsible for limiting viral clearance that could be achieved by TNFα mediated apoptosis signalling. Importantly, mice deficient in cIAP2 and cIAP1 (in hepatocytes) were more resistant to HBV infection and were able to selectively clear the virus-infected hepatocytes quicker than WT mice (Ebert, Preston*, et al.*, 2015b). This suggested that chemical biology approaches that antagoznize cIAPs may be a way to treat and clear HBV infections.

The way cells innately regulate cIAP levels is through SMAC/DIABLO. The importance of the cIAPs in HBV infection provided the opportunity to test whether SMAC mimetics, anti-cancer compounds known to target and antagonise IAPs and promote cell death, could influence HBV infection. After

administration of the SMAC mimetic birinapant, cIAP 1 and 2 levels decreased dramatically in both naïve and HBV-infected mice (Ebert, Allison*, et al.*, 2015). The decrease also correlated to a TNFα dependent control of HBV infection in infected mice. This revealed that cIAPs are important targets for controlling intracellular pathogens.

cIAP1/2 antagonism via SMAC can also prove to be clinically useful in HIV infection. Interestingly, treatment with the SMAC mimetic SBI-0637142 resulted in re-emergence of latent HIV reservoirs (Pache *et al.*, 2015). Depleting cellular levels of cIAP during HIV infection inhibited the canonical pathway of NFKB activation and induced viral replication (Pache *et al.*, 2015). This clinically relevant discovery could be used to bait out virus reservoirs into activity and then initiate clearance with conventional anti-retroviral therapy. Such a strategy also has implications for malaria, specifically for the potential to re-activate hypnozoites in hepatocytes, allowing treatment with primaquine or tafenoquine.

The disparity of the effect of SMAC mimetics against HBV and HIV infection models can be attributed to the upregulated TNFα response specifically associated with HBV (Ebert, Preston*, et al.*, 2015b). Higher levels of TNFα and TNFR1 expression during HBV resulted in induction of apoptosis following SMAC mimetic treatment, however, during HIV infection, treatment only inhibited canonical NFKB signaling without inducing apoptosis. This phenomenon highlights the importance of TNFα responses in conjunction with SMAC mimetics when considering therapeutic avenues against infectious diseases.

5.5 Inhibition of apoptosis during *Plasmodium* infection

The malaria parasite life cycle comprises multiple forms and stages within the human host. The exo-erythrocytic form (or liver stage) involves invasion and development within a nucleated human cell that features apoptotic signalling mechanisms. It is likely that the parasites interact with their infected hepatocyte during infection to inhibit host apoptotic signalling responses that normally would clear the infection in order to ensure survival.

Hepatocytes infected with *P. berghei* develop a resistance to apoptosis that is not seen in uninfected cells. Infected hepatocytes treated with molecules that induce apoptosis, such as TNFα, cycloheximide and t-butyl-hydroperoxide, show reduced signs of apoptosis compared with uninfected cells (van de Sand *et al.*, 2005). This demonstrates that exoerythrocytic forms actively protect their infected cell from apoptosis. The mechanism underlying this apoptotic resistance has been attributed partially to HGF (hepatocyte growth factor) release during sporozoite traversal events and activation of the PI3-kinase/Akt signaling pathway. However, data indicating resistance to apoptosis even during later stages of liver development, when HGF is not present, suggest that there must also be additional parasitic mechanisms that impact host cell apoptotic signaling (Leirião *et al.*, 2005). In addition to this, more recent evidence suggests that HGF signaling is unique to *P. berghei* and is not found in *P. falciparum* (Kaushansky & Kappe, 2011).

More recently, protein microarray data during malaria liver stage infections show increases in levels of Akt and bcl-2 in infected cells (Kaushansky, Ye*, et al.*, 2013). Both of these proteins are involved in pro-survival cell signalling. Levels of p53, a pro-apoptotic molecule, appeared to diminish during parasite liver infection. Furthermore, mice overexpressing p53 gained some protection against parasite liver infection, whereas mice lacking p53 showed an increase in parasite load during infection (Kaushansky, Ye*, et al.*, 2013), demonstrating the importance of regulating apoptosis during infection.

Since apoptosis plays a significant role in *Plasmodium* liver stage infection, methods to target cell signalling pathways to trigger apoptosis may prove useful in host-based prophylaxis aimed at clearing liver stage infections. The use of p53 agonists and bcl-2 inhibitors against *P. yoelii* liver stages has shown to eliminate parasites both *in vivo* and *in vitro* (Douglass *et al.*, 2015). Combination treatments targeting both p53 and bcl-2 are more effective at reducing liver stage burden than targeting either pathway individually. Mice treated with these compounds show a significant delay in establishing blood stage infection following *P. yoellii* liver stage infection (Douglass *et al.*, 2015).

Therapeutic strategies targeting apoptosis show promise with regard to killing malaria liver stages, however, only the intrinsic and p53-induced apoptotic pathways have been targeted so far. Whether the malaria parasite has any interaction with the extrinsic pathway, or if cIAPs play a role in liver stage infection is yet to be determined and may provide a new avenue for effective liver stage anti-malarial treatments, especially as targeting the host would circumvent parasite drug resistance and would provide opportunities for the host to develop immunity against the malaria parasite.

Aims of this dissertation

Knowledge of protein export and host cell modification by malaria parasites is largely limited to erythrocytic stages of infection. Genetic ablation of plasmepsin V, a key mediator of protein export, could not be achieved, revealing a crucial role for plasmepsin V in blood stage infection.

The aims of this PhD thesis are:

1. To conditionally regulate plasmepsin V expression during liver stage infection to assess the importance of protein export.
2. To further our understanding of protein export in the rodent malaria *P. berghei*, including analysing the importance of the PEXEL motif, by characterising the PEXEL containing protein, PBANKA_0700700.
3. To explore the effect of malaria liver infection on host hepatocyte cIAPs and apoptosis, dissecting potential parasite-host interactions during liver infection.

Chapter 2: Methods

Ethics

All experiments involving mice were conducted in accordance with the regulations of the National Health and Medical Research Council and were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC2014.030). The use of human erythrocytes for *P. falciparum* parasite culturing was approved by the Walter Eliza Hall Human Research Ethics Committee and the Australian Red Cross Blood Service Agreement (11-09VIC-01).

P. berghei ANKA maintenance and transfections

Swiss mice (4-6 weeks old) were housed at 22-25 C on a 12 hour light dark cycle prior to infection. Parasites thawed from cryopreserved vials were injected via intraperitonial inoculation to a donor mouse. Parasitemia was monitored by Giemsa smears. When infections reached >4% parasitemia, infected blood was collected via cardiac puncture in a heparinised syringe.

Transfections in *P. berghei* ANKA were performed using methods described by Janse (2006). Infection was established in Swiss acceptor mice, which were monitored daily for 2-3 days post infection until a parasitemia of 0.5-3% was achieved. Infected blood was collected via cardiac bleed and parasites were cultured overnight in "Schizont culture media" containing RPMI1640 with HEPES (Sigma), $NaHCO_3$ (2g/L), 50,000 I.U. neomycin trisulfate with pH modified to 7.3. Media was supplemented with 20% heat-inactivated Fetal Calf Serum (HI-FCS; Gibco) prior to parasite culture. Heat inactivation was achieved by incubation at 60˚C for 30 min. Overnight incubation of *P. berghei* in Schizont

culture media at 37˚C results in the formation of mature schizonts that are unable to rupture. This enables the enrichment of schizonts for downstream use.

Mature schizonts were purified from these cultures using magnet purification. The parasites were passed slowly (dropwise) through a commercial MACS CS separation column (Miltenyi Biotec) fixed upon VarioMACS magnet assembly. Mature schizonts contain an iron-haemozoin crystal causing these parasites to be trapped within the column as media is passed through due to the magnetic field from the VarioMACS magnet. Parasites were eluted in the absence of the magnet in incomplete media lacking HI-FCS.

Transfections were performed using the Amaxa Nucleofector device (Lonza, program setting U33) and Basic Parasite Nucleofector Kit 2 (Lonza). Transfected parasites were injected into 4-6 week old Swiss mice by intravenous injection with 27G needles. Selection for transgenic *P. berghei* involved using either *hDHFR* or *TgDHFR* resistance cassettes. Both selection cassettes confer resistance to pyrimethamine, which was administered orally (70 mg/ml) via the drinking water (pH 3.5-4) to infected mice from 1 day post-infection. When the parasitemia in infected mice reached >1%, blood was harvested and either used for storage in liquid nitrogen, as an inoculum in subsequent mice, to prepare genomic DNA (gDNA) for genotypic analysis or to prepare samples for protein expression analysis by immunoblot.

Preparation of DNA for cloning

The PMV-Flp FRT parasite line and PBANKA_0700700 WT and PEXEL Mutant parasite lines were generated using synthesized gene sequences from Life technologies. Flanks were cloned into the PBANKA plasmid pb3 (de Koning-Ward *et al.*, 2000), modified with human dihydrofolate reductase (DHFR) instead of *Toxoplasma gondii* DHFR. Cloning into this vector used the restriction sites ApaI and ClaI. Transfections for the PMV-Flp line were performed using a modified ANKA strain TRAP-FLP expressing parasites as described initially in La

Croix *et al.* (2011). Transfections of the PBANKA_0700700 lines were done using wild type PBANKA.

Western Blotting

Samples were processed in reducing sample buffer (Invitrogen) containing 125mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS (Sodium Dodecyl Sulfate) and 5% β-mercaptoethanol using SDS PAGE (Polyacrylamide gel electrophoresis) 4-12% Bis/Tris Invitrogen gels. Gels were electrophoresed at 140V for 1.5 hours in running buffer. Gels were transferred to nitrocellulose membrane using transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 100 V for 1 hour. Nitrocellulose membranes were blocked for at least 1 hour in 10% (v/v) skim milk dissolved in 0.05% Tween 20. Membranes were then incubated with primary antibodies (anti-rabbit HA antibody, Sigma) , diluted with 1% skim milk 0.05% Tween 20 and binding was detected with secondary anti-mouse or anti-rabbit horse radish peroxidase (HRP)-conjugated antibodies (1:1000) HRP-Antibody binding was detected with incubation with chemiluminescent substrate (Amersham ECL reagent, GE healthcare sciences) and exposure to autoradiography film.

Immunofluorescence microscopy

For both *P. falciparum* and *P. berghei*, asynchronous infected RBCs with parasitemia ranging from 1-5% were smeared and air-dried for 1 hour. Slides were fixed in ice-cold 100% methanol for 3 min and stored at -80^{o}C until use. Parasites were probed with rabbit polyclonal IgG (Sigma) reactive to the Human Influenza hemagglutinin (HA) epitope (YPYDVPDYA) diluted 1 in 1000 in a PBS 1% BSA solution. Following 3 washes with PBS (5 min each), secondary antibody anti-rabbit Alexa 488 (1 in 1000, Invitrogen) was added for 30 min. After an additional wash step, parasites were mounted with Vectashield (Vector Laboratories) and 0.1ng/µl 4', 6-diamino-2-phenylindole, DAPI (Invitrogen).

All micrographs were collected on a Deltavision Elite microscope with an Olympus 100x UPlanSApo NA1.4 objective using SoftWorx software. Images were captured using CoolsnapHQ2 charge-coupled device (CCD) camera and analysed with ImageJ Fiji (v.1.45b). Maximum projections were taken using the average of multiple Z-stacks.

Fluorogenic PEXEL cleavage assays

PEXEL cleavage assays were prepared as previously described (Boddey *et al.*, 2010). Reactions were performed in 396 well plates in 20 ul total volume and consisted of 0.2ul Plasmepsin V (*P. berghei* and *P. vivax*) conjugated-agarose, digest buffer (25 mM Tris, 25 mM MES, pH 6.4) and 1.5 mM FRET peptide substrate containing either a WT PEXEL sequence from the exported protein, knob-associated histidine-rich protein (KAHRP) or PEXEL point mutations (R>K, L>I or RL>A). The peptide substrates were synthesised by Mimotopes or LifeTein and the sequences were as follows. WT: DABCYL-RNK**RTLAQ**KQ-E-EDANS; R>K: DABCYL-RNK**KTLAQ**KQ-E-EDANS; L>A: DABCYL-RNK**RTIAQ**KQ-E-EDANS; RL>A: DABCYL-RNK**ATAAQ**KQ-E-EDANS, LifeTein). Samples were incubated at 37 C to allow the reaction to proceed and substrate cleavage measured by excitation at 340 nm and reading fluorescence emission at 492 nm using an Envision fluorescence plate reader (Perkin-Elmer). Measurements were taken regularly at hourly intervals.

P. berghei mosquito infections

Swiss mice 4-6 weeks old were injected intraperitoneally with 300-350 ul packed blood from an infected donor mouse (4% parasitemia). When the parasitemia reached 3-5% with a gametocytemia of >1%, the mice were anaesthetised with an intraperitoneal injection of ketamine at 400ul/20g, and then directly fed to 3-5 day old *Anopheles stephensi* mosquitoes (Johns Hopkins University) for 15 min. Mice were culled post infection and the mosquitoes were

stored at 21˚C with 70% humidity and were glucose-starved overnight to select for only blood-fed mosquitoes. Surviving mosquitoes were provided sugar cubes and water (via cotton wick) *ad libitum.*

Mosquitoes midguts were dissected 14 days post infection to quantify oocysts. Midguts were stained with 0.1% mercurochrome for 15 min and then mounted on a slide to count oocysts. Salivary gland sporozoites were dissected and quantified on days 19-23 post infection for the isolation of salivary gland sporozoites. Salivary glands were kept on ice in RPMI 1640 with HEPES (Sigma) and $NaHCO_3$ and were crushed in eppendorf tubes with a pestle to liberate sporozoites within an hour of dissection.

In the case of the PMV-Flp parasites, P. berghei infected mosquitoes were stored in conditions as described by La Croix *et al* (2011). Importantly, the temperature the mosquitoes were kept at was room temperature (21˚C) for the first 16 days post infection. On day 16, the temperature was raised to 25°C to initiate flp activity and promote the excision to take place.

Quantitative Real-Time PCR liver sample preparation

Four to six week old C57Bl/6 mice were intravenously injected with 10,000 sporozoites freshly dissected from mosquitoes. At the appropriate time points (6h, 24h, 44h), the infected mice were euthanized using CO_2 and liver lobes were isolated by dissection. Lobes from an individual mouse were emulsified into a single cell suspension using a cell strainer and pestle. Liver cell suspensions (300 µl) were added to 800 µl TriZol (Thermoscientific) and frozen at -80°C until RNA extraction was to be performed.

For RNA extraction of samples, 200 µl of chloroform was added, then samples were centrifuged at 12,000 g for 15min at 4°C. RNA is present in the aqueous phase and was carefully pipetted into another eppendorf tube without disturbing the interphase. RNA was then purified using isopropanol (500 µl)

precipitation and then washed with 75% ethanol (1ml). The RNA pellet was then dissolved into DEPC-treated water (Thermoscientific). RNA was reverse transcribed to cDNA using random oligo dt oligonucleotides and the protocol described within the Qiagen Quantitect Reverse Transcription kit.

Real time PCR analysis was performed using protocols described within Gomes-Santos *et al.* (2011), using SYBR Green (Applied Biosystems) according to the manufacturers instructions. Liver infection load was quantified using qRTPCR analysis of *P. berghei* 18S rRNA normalized against hypoxanthine-guanine phosphoribosyltransferase (HPRT) using previously described primers (Gomes-Santos *et al*., 2011).

Hepatocyte culture

HepG2 cells were maintained in T75cm^2 vented cap flasks (Corning) using DMEM (Dulbecco's Modified Eagle Media) supplemented with 10% heat inactivated Fetal Calf Serum (Gibco), 100IU/ml Pen 100 µg/ml Strep (Sigma) at 37°C in 5% CO_2. Once cells reached high confluency (~90%, approximately every 3 days) they were split 1:4. The hepatocyte monolayer was washed with PBS and then pre-warmed 0.05% Trypsin-EDTA solution was added for 5 minutes. After the cells were dispersed, the trypsin was removed by adding an equal volume of complete growth media.

LISP2 Immunofluorescence Assay

Salivary gland sporozoites (10,000) dissected on day 21-23 were added to 50,000 HepG2 cells (seeded onto collagen coated cover slips 16 hours prior). At specified timepoints following infection, the cells were fixed using 4% paraformaldehyde for 20 minutes and then washed and kept in PBS. Primary antibodies in 3% BSA, 1:1000 rabbit anti-LISP2 (Orito *et al.,* 2013) and 1:500 chicken anti-EXP1, were then added to the fixed cells. Secondary antibodies used were goat anti-rabbit Alexa 488 and goat anti-chicken Alexa 594. Images were

collected on a Deltavision Elite microscope with an Olympus 100x UPlanSApo NA1.4 objective using SoftWorx software. Images were captured using CoolsnapHQ2 charge-coupled device (CCD) camera and analysed with ImageJ Fiji (v.1.45b).

Statistics

All statistical analyses (generation of p-values) were conducted using Graphpad Prism 6. The Mann-Whitney non-parametric test and standard t-tests were used.

Chapter 3: Plasmepsin V is essential for liver stage development of *Plasmodium berghei*

Summary

During erythrocytic infection, malaria parasites remodel the host cell through the export hundreds of effector proteins across the parasitophorous vacuole membrane into the host cell. Export of most proteins requires cleavage of the *Plasmodium* export element (PEXEL) by the aspartyl protease, plasmepsin V (PMV). Prior to blood stage infection, malaria parasites infect the liver. The importance of the PEXEL motif and plasmepsin V in liver stages is uncertain. Employing conditional mutagenesis to excise the plasmepsin V locus during passage through mosquitoes, we show in *Plasmodium berghei* that plasmepsin V plays an essential role in liver stage development. Export of the essential liver stage protein, Liver-specific protein 2 (LISP2), is also defective in plasmepsin V mutant parasites, with the protein accumulating within the endoplasmic reticulum and parasitophorous vacuole of exoerythrocytic forms. A PEXEL motif located within LISP2 is processed by plasmepsin V in a biochemical assay and mutations of the PEXEL arginine and leucine residues abolish processing. Furthermore, arrest of plasmepsin V-deficient exoerythrocytic parasites provides protective immunity in mice against a lethal challenge of sporozoites. Together, these data reveal that plasmepsin V is essential in liver stage parasites and facilitates export of an effector protein to the infected hepatocyte. This suggests that components of PEXEL-mediated export are conserved across both the blood and liver stages of the *Plasmodium* lifecycle.

Introduction

Malaria is devastating disease caused by parasites of the genus *Plasmodium*. The malaria parasite lifecycle consists of multiple replication steps within the human host, including parasite forms that infect both liver cells and red blood cells. It is the erythrocytic stage of infection that features an extraordinary process of cellular remodelling involving parasites exporting proteins across an encasing vacuole membrane into the host cell. This remodelling process produces a rigid and cytoadherent infected cell, allowing the parasite to evade host immune responses, causing the clinical manifestations and pathogenesis of malaria.

Exported proteins feature an N-terminal motif called the *Plasmodium* export element (PEXEL, RxLxE/Q/D) (Marti *et al.*, 2004) or vacuolar transport signal (VTS) (Hiller *et al.*, 2004). The PEXEL is important for both ER entry and translocation across the vacuole. Cleavage of this motif occurs in the ER by the aspartyl protease plasmepsin V (Boddey *et al.*, 2010; Russo *et al.*, 2010). Attempts to disrupt plasmepsin V expression in *P. berghei* and *P. falciparum* blood stages have been unsuccessful (Boddey *et al.*, 2010; Russo *et al.*, 2010). Conditional knockdown of plasmepsin V expression and the development of PEXEL-mimetic inhibitors have subsequently shown that plasmepsin V is essential in asexual blood stages for protein export and erythrocyte remodelling (Hodder *et al.*, 2015; Sleebs *et al.*, 2014). The function of plasmepsin V is yet to be elucidated in the rodent malaria parasite, *P. berghei*. However, use of this malaria model has several advantages, including the ability to complete the lifecycle and infect mice with sporozoites in order to study the function of plasmepsin V during pre-erythrocytic stages *in vivo*. In asexual blood stages, cleaved PEXEL proteins utilise the *Plasmodium* translocon of exported proteins (PTEX) to translocate across the vacuole membrane and enter the host erythrocyte (Beck *et al.*, 2014; de Koning-Ward *et al.*, 2009; Elsworth *et al.*, 2014).

In liver stages of infection, the extent to which the parasite remodels the host cell is less clear. Only two proteins have been previously confirmed to be exported

into the hepatocyte cytoplasm. One study demonstrated the export of circumsporozoite protein (CSP) in *P. berghei* using GFP fusions of the CSP PEXEL (Singh *et al.*, 2007). CSP was described to localise to the hepatocyte cytoplasm and mutation of the two PEXEL motifs located within the CSP N-terminus resulted in confinement of the mutant protein within the parasite. However, subsequent studies have shown that CSP containing either native or mutated PEXELs can be transported beyond the parasitophorous vacuole (Cockburn *et al.*, 2011), suggesting that the PEXEL may not be directing export of CSP in infected hepatocytes. Another exported protein in the *Plasmodium* liver stage is 'liver specific protein 2 (LISP2)'. LISP2 is one of the most abundantly expressed *P. berghei* liver stage proteins and, using immunofluorescent labelling, was detected in the hepatocyte cytoplasm during late liver stage infections (Orito *et al.*, 2013). The authors noted the presence of a PEXEL-like motif RILAE in LISP2, but that it is located within an unusual location in the protein, 370 amino acids from the N-terminus. They suggested that the PEXEL-like sequence was not conserved in LISP2 from other *Plasmodium* spp. and may not contribute to the export of the protein (Orito *et al.*, 2013). Interestingly, conjugation of a PEXEL motif to a truncated ovalbumin peptide caused an increase in T-cell recognition and proliferation in liver cells when compared with the T-cell recognition against a peptide lacking the PEXEL motif (Montagna *et al.*, 2014). This is indirect proof of protein export regulated by parasites to evade immune recognition during the liver stage. The molecular mechanisms of export and functionality of the PEXEL motif in liver stage infections, as well as the positional constraints of such a sequence in liver stage proteins, still remains uncertain.

In this study, we show that the role of plasmepsin V is conserved in *P. berghei* blood stages through the identification of a new PEXEL-containing protein, PBANKA_0700700 that is exported to punctate structures in the infected murine erythrocyte. Using the *flp*-FRT conditional transgenesis system previously described by Lacroix *et al.* (2011), we reveal the crucial role plasmepsin V plays in liver stage infection. We show that plasmepsin V mutants arrest by 24 hours post-infection and that LISP2 export is abrogated in the plasmepsin V mutant. We also show that LISP2 in all *Plasmodium* spp. possesses a conserved PEXEL

motif and is cleaved by plasmepsin V in a biochemical assay. Tagging of LISP2 with mCherry prevents its export, and the PTEX unfoldase component HSP101 is apparently absent from the liver-stage translocon, indicating that this tag interferes with export. These observations suggest that some features of the export pathway are conserved across the blood and liver stages of malaria infection, although the positional constraints of the PEXEL for export in blood stages appears to be less strict in exo-erythrocytic forms and the fusion of mCherry to the N-terminus apparently blocks export in hepatocytes but not in erythrocytes, possibly owing to differences in PTEX composition.

The novel PEXEL protein PBANKA0700700 is cleaved by plasmepsin V and exported to the *P. berghei*-infected RBC

Export of PEXEL-containing proteins to the infected erythrocyte has been reported previously with *P. berghei* (Haase *et al.*, 2013; Ingmundson *et al.*, 2012; MacKenzie *et al.*, 2008; Petersen *et al.*, 2015), however, whether the PEXEL is cleaved, and the role of plasmepsin V, is yet to be determined in this parasite species. To examine this, we selected a previously uncharacterised PEXEL protein with no known function for further study, called PBANKA_0700700. This gene is transcribed in asexual and liver stages (in accordance with data from PlasmoDB) and was chosen for further analysis because it contains a PEXEL motif (RILSS) and a knockout parasite line had been generated by our colleague, Dr Blandine Franke-Fayard, who generously shared this unpublished mutant with us for subsequent characterization. To study the export of PBANKA_0700700, it was endogenously tagged with 3x hemagglutinin (HA) tags at the C-terminus by double crossover homologous recombination, so that expression was driven from the endogenous gene promoter (Figure 1A). A control *P. berghei* parasite line was generated using the same approach in which a mutant PEXEL (AIASA) form of PBANKA_0700700 was tagged with 3xHA as a control (Figure 1B).

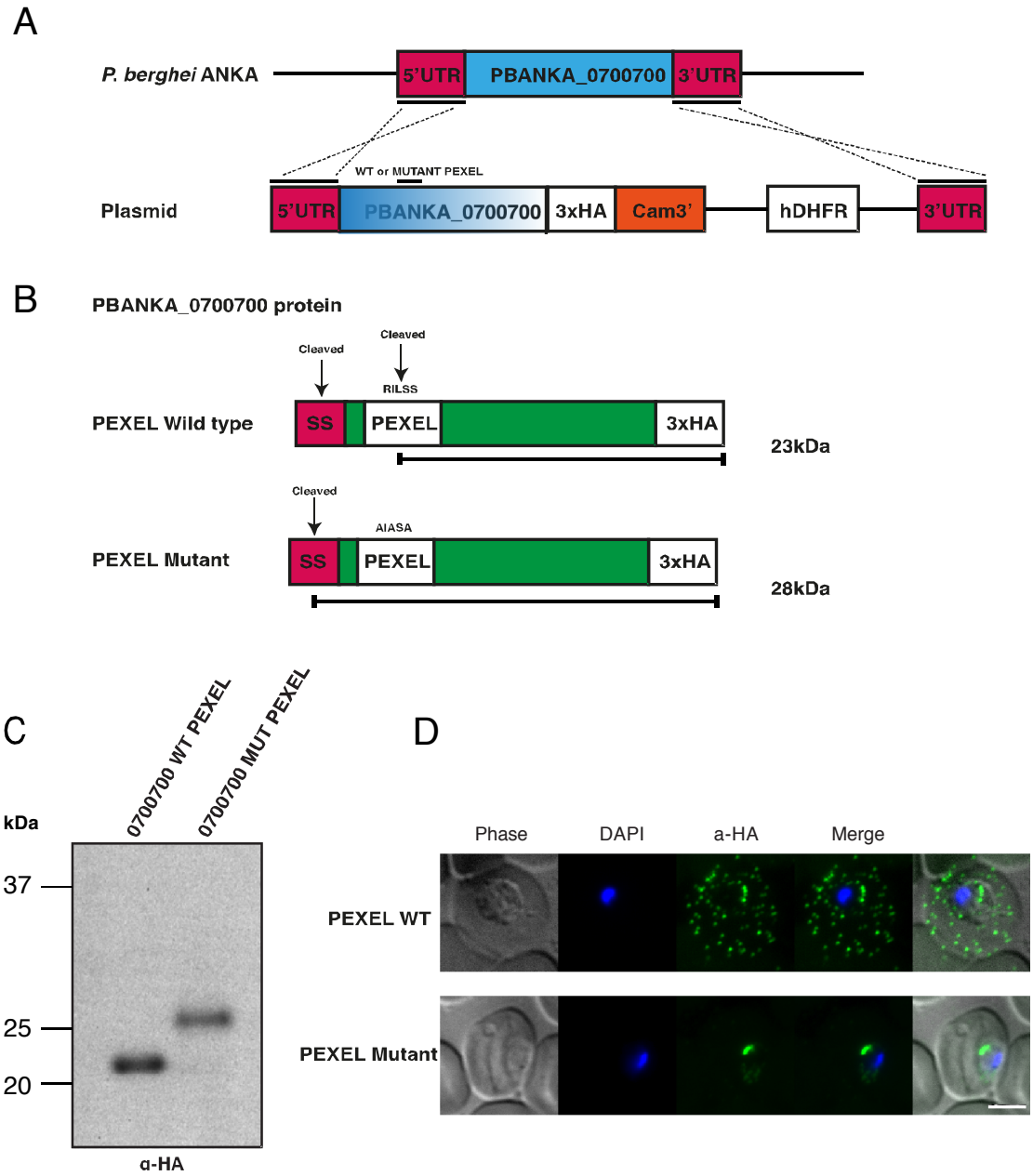


Figure 1: PBANKA_0700700 is an exported PEXEL-containing protein.
A. Plasmid schematic showing the genetic replacement of the endogenous PBANKA_0700700 gene with a 3xHA tagged codoned complement. Plasmids were generated with both a Wild type (RILSS) and Mutant PEXEL (AIASA). **B.** Diagram indicating the expected N-terminus processing sites and resulting protein sizes of PBANKA_0700700. **C.** Western blot analysis of infected RBCs with both WT and mutant PEXEL PBANKA_0700700 parasites. PBANKA_0700700 undergoes processing at the PEXEL motif, causing a mature protein size of 23 kDa. PEXEL processing is abolished in the PEXEL mutant line, resulting a larger protein species (28 kDa). **D.** Immunofluorescence analysis of ring-stage infected mouse RBCs. Anti-HA labelling (green) shows export of the protein into the erythrocyte cytoplasm for the WT PEXEL. The mutated PEXEL is trapped within the parasite. Scale =2 μm.

In *P. falciparum*, protein export is preceded by processing of the PEXEL motif. To assess potential N-terminal processing of PBANKA_0700700, parasites were collected from mouse blood lysates and analysed by immunoblot using anti-HA antibodies. The PBANKA_0700700 PEXEL WT migrates as a smaller protein species (23kDa) than the protein with a mutant PEXEL (28kDa) (Figure 1C), indicating that the native PEXEL is indeed being processed in the parasite. To investigate export of PBANKA_0700700-HA to the infected erythrocyte, immunofluorescence microscopy was performed on fixed *P. berghei* infected mouse red blood cells. The processing result seen from the immunoblot correlates to the localisation of the protein, as the WT PEXEL fusion is exported into the host erythrocyte (Figure 1D). Its localisation was observed in discrete intra-erythrocytic *P. berghei* induced structures (IBIS) similar to that of the previously described IBIS1 protein (Ingmundson *et al.*, 2012; Petersen *et al.*, 2015). IBIS structures functionally appear identical to the Maurer's clefts described in *P. falciparum* (Petersen *et al.*, 2015). The PBANKA_0700700 PEXEL mutant does not share the same localisation, however, as the uncleaved PEXEL species is trapped within the parasite itself and is not exported. These observations show the PEXEL motif is cleaved in an exported *P. berghei* protein and that mutation of the conserved PEXEL residues abolishes processing and export. This is similar to the function and specificity of the PEXEL for export in *P. falciparum* (Boddey *et al.*, 2009; Chang *et al.*, 2008; Hiller *et al.*, 2004; Marti *et al.*, 2004), demonstrating that the motif is functionally conserved between rodent and human malaria parasites. This strongly suggests that plasmepsin V (Boddey *et al.*, 2010; Russo *et al.*, 2010) has a conserved function in both species.

The function of plasmepsin V is conserved in *P. berghei* blood stages

To study the localization and function of plasmepsin V in blood stages, it was HA-tagged (see *flp*-FRT system below and Figure 2A). Western blot analysis, diagnostic PCRs and immunofluorescence assays all confirmed integration of the *PMV-HA-FRT* locus and expression of PMV-HA in blood stages with a perinuclear localisation, suggestive of an ER localization, similar to plasmepsin V in *P. falciparum* (Boddey *et al.*, 2010; Russo *et al.*, 2010)(Figure 2B, C). PbPMV-HA was affinity purified using anti-HA agarose beads, as previously described for plasmepsin V from *P. falciparum* and *P. vivax* (Boddey *et al.*, 2010; Boddey *et al.*, 2013; Sleebs *et al.*, 2014). The activity of purified *P. berghei* plasmepsin V was assessed in a fluorogenic PEXEL cleavage assay developed previously (Boddey *et al.*, 2010; Boddey *et al.*, 2013; Sleebs *et al.*, 2014) incubated with a fluorogenic substrate consisting of the PEXEL sequence from the exported PEXEL protein knob associated histidine rich protein (KAHRP). Efficient processing of the KARHP PEXEL was observed by *P. berghei* plasmepsin V (Figure 2D). Mutation of the conserved Arg and/or Leu residues (R>K, L>I, 2A) of the PEXEL resulted in complete abrogation of processing by *P. berghei* plasmepsin V (Figure 2D). This result indicates that plasmepsin V cleaves the PEXEL motif with the same substrate specificity as shown for *P. falciparum* and *P. vivax* plasmepsin V (Sleebs *et al.*, 2014).

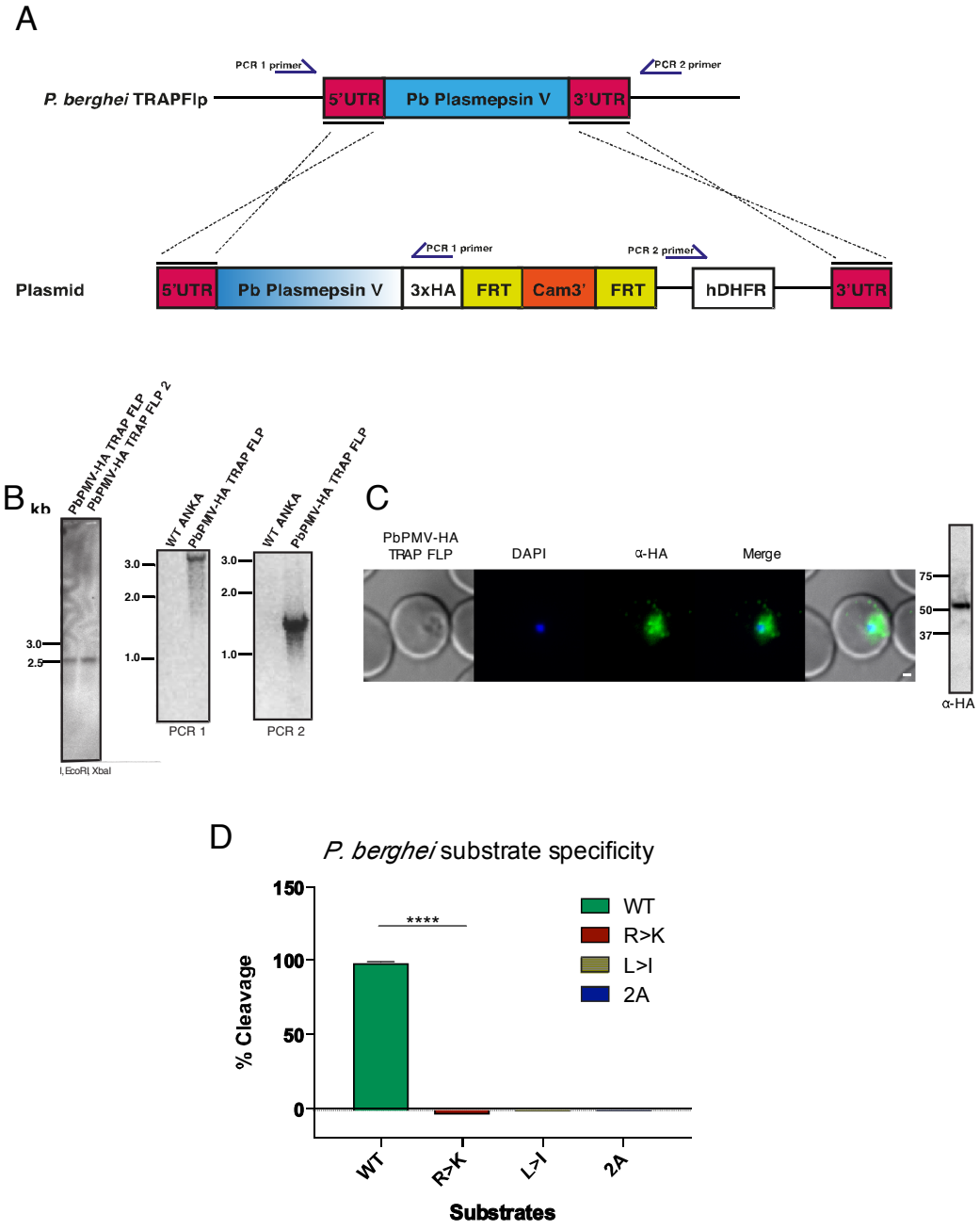


Figure 2: Generation and confirmation of the *P. berghei* plasmepsin V FRT conditional knockdown. **A.** Plasmid schematic showing the homologous recombination event used to generate the PMV TRAP-Flp line. The endogenous PbPMV gene was tagged with 3xHA and the PbCam3'UTR was integrated with FRT sites flanking it. **B.** Southern blot and PCR analysis confirming correct integration of the line. PCR primer binding sites are shown in A. **C.** Immunofluorescence of ring-stage parasites from mouse RBC showing plasmepsin V expression (anti-HA, green) and localisation to the endoplasmic reticulum. Scale = 2 µm. Western blotting (anti-HA) confirmed expression of Ha-tagged plasmepsin V) **D.** *In vitro* incubation of peptide substrates containing the PfKARHP PEXEL motif with immunopurified PbPMV shows strong cleavage of the WT PEXEL. This suggests PbPMV has highly specific activity for the PEXEL motif.

Plasmepsin V is essential in liver stages

So far, all attempts to delete the *plasmepsin V* gene in *P. falciparum* and *P. berghei* have been unsuccessful (Boddey *et al.*, 2010; Klemba & Goldberg, 2005; Russo *et al.*, 2010). To study the function of plasmepsin V in liver stages, we employed the *flp*-FRT conditional mutagenesis system in *P. berghei* to conditionally delete the PMV locus during mosquito passage (Lacroix *et al.*, 2011). The *flp*-FRT system uses flippase (*flp*) recombinase from *Saccharomyces cerevisiae* (Zhu & Sadowski, 1995) that recognizes a pair of *flp* recombinase target (FRT) sequences flanking the locus of interest. In this way, native plasmepsin V expression can be retained in blood stages, but stage-specific excision of the *plasmepsin V* locus occurs during mosquito passage in developing oocysts, providing mutant sporozoites for subsequent analysis in liver stage infections. The *plasmepsin V* gene-targeting construct was designed so that, upon integration, the native 3' UTR would be replaced with the *P. berghei CAM* gene 3'UTR flanked by FRT (GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC) sequences (Figure 2A). A 3xHA tag was also incorporated at the 3' of the *plasmepsin V* gene to allow detection of the gene product with commercial anti-HA antibodies. This plasmid was transfected into *P. berghei* that already expresses *flp* recombinase from the *thrombospondin related anonymous protein* (*TRAP*) gene promoter (Lacroix *et al.*, 2011), which is expressed only during sporozoite stages of the lifecycle. Excision of the CAM 3' UTR results in unstable mRNA lacking the poly adenine tail, causing its degradation and loss of protein levels. Transfectants were generated and confirmed via diagnostic PCR, immunofluorescence assays and western blot analysis (Figure 2B, C).

The PMVHA-FRT/Flp transgenic line was analysed for its efficiency of genomic deletion. PCR analysis of genomic DNA (gDNA) prepared from asexual blood stages prior to mosquito passage generated a PCR product of 1.7 kb (see PCR 1, Figure 3A, B). The same PCR reaction performed on gDNA subsequently isolated from sporozoites in the mosquito salivary glands produced a 1.3 kb product, indicating near complete excision of the *PMV-CAM* 3' UTR had occurred (see PCR 1, Figure 3A, B). In sporozoites, no amplicon was detected using an

oligonucleotide pair for which one binds within the *PMV-CAM* 3' UTR, whereas the expected 1.4 kbp amplicon was detected from asexual blood stages prior to mosquito passage (see PCR 2, Figure 3A, B). Collectively, these results are consistent with highly efficient excision of the *PMV-CAM* 3' UTR in salivary gland sporozoites.

The PMVHA-FRT/Flp line was then tested for its ability to establish patent infections in Swiss mice following intravenous (i.v.) injection into the tail vein. 10,000 salivary gland sporozoites of either the PMVHA-FRT/Flp transgenic line or a WT ANKA *P. berghei* strain (control) were injected intravenously into mice. Interestingly, all 33 mice infected with PMVHA-FRT/Flp sporozoites failed to produce a patent blood stage infection, whereas WT ANKA control parasites initiated patent infections in all 22 mice that were infected (Table in Figure 3C). The lack of patent infections following injection with PMVHA-FRT/Flp sporozoites indicates a defect occurred either in sporozoite invasion, liver stage development and/or initiation of blood stage infection.

To examine the time of mutant parasite arrest, parasite load in mouse livers infected with PMVHA-FRT/Flp or WT ANKA *P. berghei* sporozoites was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) at 6 h, 24 h and 44 h post infection. The 6 h time point revealed no significant difference in parasite liver load between WT and PMV mutant parasites, suggesting the mutants were able to navigate to and establish early liver infections equally to that of WT. However, a dramatic decrease in parasite liver load for the PMV mutant was observed at both the 24 h and 44 h time points in comparison to WT, the latter of which amplified in number exponentially as expected (Figure 3D). These results suggest a significant defect in liver stage development for PMVHA-FRT/Flp parasites. Together with the patency data above, this reveals that PMV is essential for liver stage development in *P. berghei*. A small, albeit detectable, parasite liver load was observed in some mice at 44 h timepoints (Figure 3D). The complete lack of patent infections in all mice suggests either that such parasites could not fully complete exoerythcytic development or that, if they

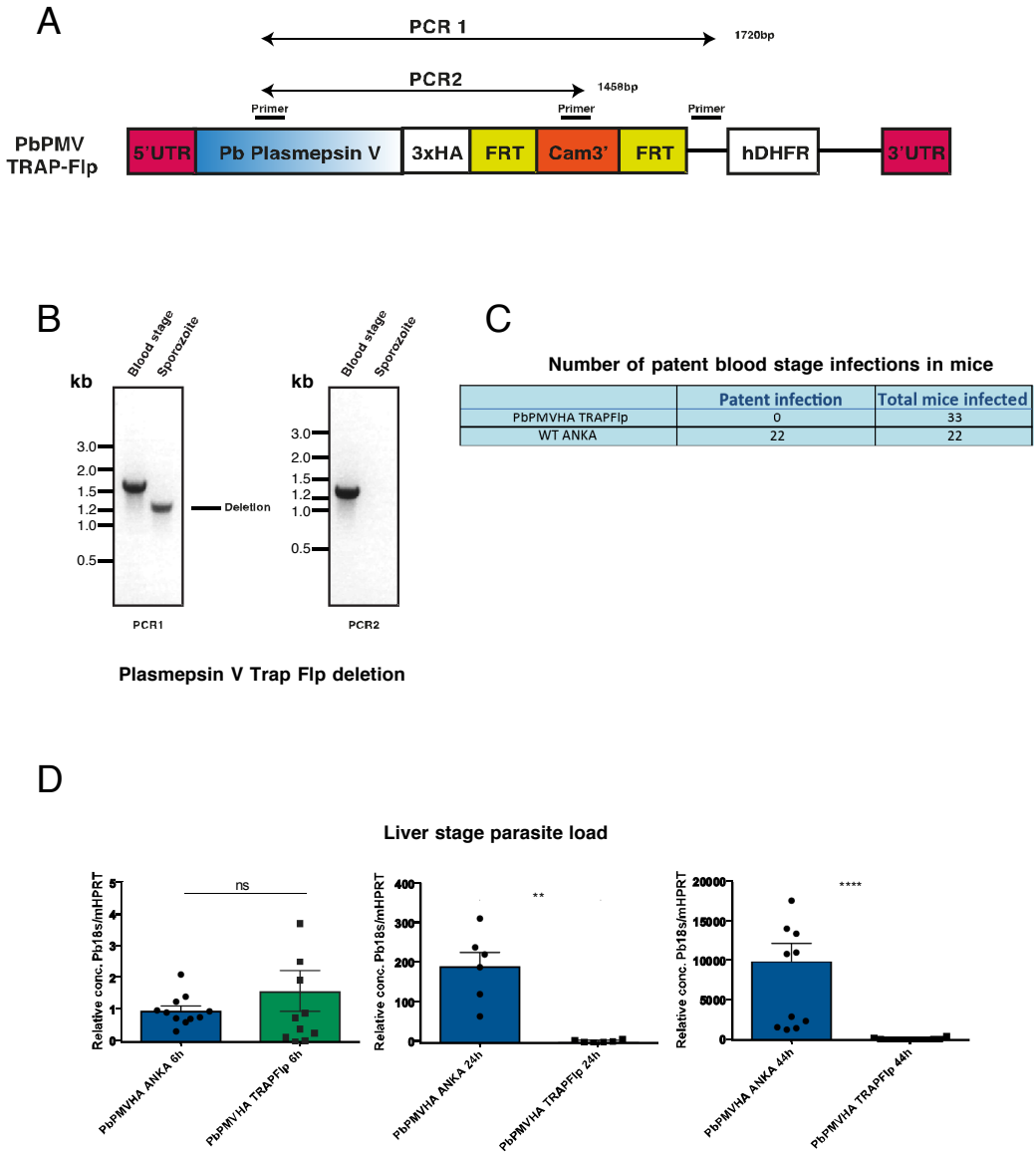


Figure 3: A, B. Schematic of the parasite genome at the PMV locus for the PMVHA-FRT/Flp line with PCR analysis of gDNA from blood stage parasites and sporozoites confirming deletion of the 3'UTR. PCR 1 amplifies across the entire region of deletion and a band of 1458 bp in sporozoite gDNA suggests the deletion has occurred. PCR 2 features a primer amplifying within the deleted 3'UTR. The lack of amplification in spozoroite gDNA suggests the 3'UTR is no longer present. **C.** Table showing patency to infection in Swiss mice injected with 10,000 sporozoites for each line. **D.** Quantification of liver parasite load using RT-PCR in mouse livers at different time points following 10,000 sporozoite intravenous injection. Data represents individual mice combined from 2 different experiments for time points 6h and 44h and one experiment for 24h (n=6-12).

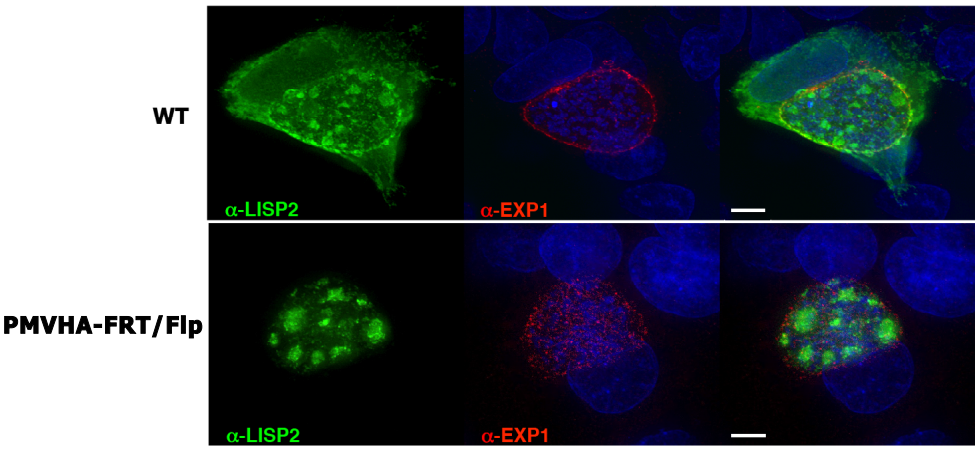
could, they died during the asexual blood stage before parasitemia could be detected.

LISP2 export is defective in PMVHA-FRT/Flp parasites

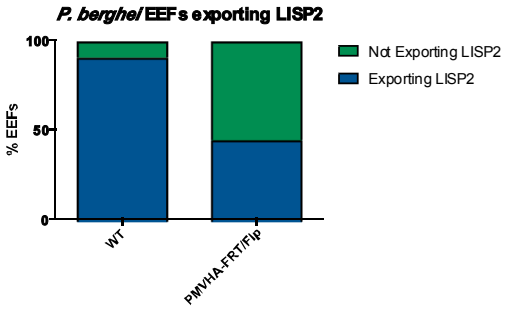
To further understand the role of plasmepsin V in liver stages, we studied a previously identified liver stage protein exported by *P. berghei* into the infected hepatocyte, called liver-specific protein 2 (LISP2) (Orito *et al.*, 2013). Expression of LISP2 begins at 24h during *P. berghei* liver stages, but it is not until later that export can be detected (Orito *et al.*, 2013). Thus, it is not possible to detect LISP2 *in vivo* using PMVHA-FRT/Flp parasites, as they arrest too early to allow imaging analysis. It was theorised that *in vitro* hepatocyte infections may yield prolonged survival of PMVHA-FRT/Flp parasites due to the absence of the immune system, allowing for the assessment of LISP2 export at later time points.

HepG2 cells were infected with either PMVHA-FRT/Flp or WT ANKA *P. berghei* sporozoites and fixed at 48 h post infection. Immunofluorescence microscopy with an anti-LISP2 antibody (Orito *et al.*, 2013) was performed on fixed samples and exported protein 1 (EXP1) was used as a marker for the parasitophorous vacuole membrane. As previously reported, LISP2 expression was strong and clearly localised to the infected hepatocyte cytoplasm in most WT ANKA infected hepatocytes as well as in the parasitophorous vacuole and internal to the parasite (Figure 4a) (Orito *et al.*, 2013). Interestingly, the PMVHA-FRT/Flp parasites showed a mixed population with regard to LISP2 localisation. Over 50% of PMVHA-FRT/Flp parasites did not export LISP2, instead, the protein localised to within the parasite (Figure 4B) yet export was detected in a subpopulation of cells that likely retained plasmepsin V expression through rescued expression of plasmepsin V mRNA either with or without the polyA tail as previously described (Ecker *et al.*, 2012). This trapped localisation is restricted to the interconnected large punctate ER structures of exoerythrocytic forms reported previously in *P. berghei* liver stages (Kaiser *et al.*, 2016) as well as to the parasitophorous vacuole. Accumulation of non-exported protein within

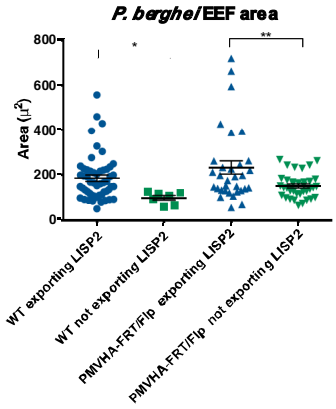
A


WT
PMVHA-FRT/Flp
α-LISP2
α-EXP1
α-LISP2
α-EXP1

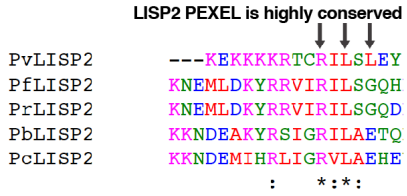
B


P. berghei EEFs exporting LISP2
% EEFs
100
50
0
WT
PMVHA-FRT/Flp
Not Exporting LISP2
Exporting LISP2

C


P. berghei EEF area
Area (μ²)
800
600
400
200
0
WT exporting LISP2
WT not exporting LISP2
PMVHA-FRT/Flp exporting LISP2
PMVHA-FRT/Flp not exporting LISP2

D


LISP2 PEXEL is highly conserved
PvLISP2 ---KEKKKKRTCRILSLEY
PfLISP2 KNEMLDKYRRVIRILSGQH
PrLISP2 KNEMLDKYRRVIRILSGQD
PbLISP2 KKNDEAKYRSIGRILAETQ
PcLISP2 KKNDEMIHRLIGRVLAEHE

E

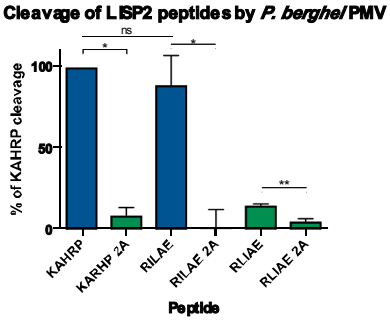

Cleavage of LISP2 peptides by P. berghei PMV
% of KAHRP cleavage
100
50
0
ns
KAHRP
KAHRP 2A
RILAE
RILAE 2A
RLIAE
RLIAE 2A
Peptide

Figure 4: Export of LISP2 in liver stage infection **A.** Immunofluorescent staining of cultured HepG2 cells 48 hours post-treatment with sporozoites. Infected cells were stained fixed and stained with anti-LISP2 (green) and the vacuole marker anti-EXP1 (red). **B.** Counts of infected hepatocytes of both WT and PMVHA-FRT/Flp parasites. Cells were divided into those that exported LISP2 and those that did not. The PMVHA-FRT/Flp parasites exhibited a clear defect in ability to export LISP2. **C.** Individual sizes (area) for each of the cells counted were also measured. Findings indicate no significant size difference between WT parasites that exported LISP2 and PMVHA-FRT/Flp parasites that did not export LISP2, suggesting that the lack of LISP2 export for the PMVHA-FRT/Flp line was not due to decreased size, but genetic ablation of plasmepsin V. **D.** Alignment of LISP2 from several species of *Plasmodium* with the region surrounding the presumed PEXEL motif (RILAE). **E.** Peptides featuring this motif were incubated with *P. berghei* plasmepsin V purified from parasites in an *in vitro* cleavage assay. This motif appears to be a functional PEXEL, however, the PEXEL-like repeat sequence, RLIAE, did not show significant processing.

the ER and parasitophorous vacuole of infected hepatocytes is similar to the location of uncleaved PEXEL mutant proteins in infected erythrocytes (Boddey *et al.*, 2010; Boddey *et al.*, 2013; Boddey *et al.*, 2009; Marti *et al.*, 2004; Russo *et al.*, 2010).

The size (area) of exoerythrocytic forms in each examined infected hepatocyte was also measured (Figure 4C). Parasites exporting LISP2 were larger in size independent of parasite strain than those not exporting LISP2. This result is expected as LISP2 export is detected as parasites mature during liver stage infection (Orito *et al.,* 2013), hence smaller or less developed parasites are less likely to export LISP2 (Figure 4C). This means that the lack of LISP2 export in the PMVHA-FRT/Flp group is due to the plasmepsin V deletion and not solely because of poor parasite development.

LISP2 is cleaved by PMV in a PEXEL-dependent manner

LISP2 export is defective in PMVHA-FRT/Flp parasites, which suggests that LISP2 may possess a cleavage site for plasmepsin V. In blood stages this is defined by the presence of a PEXEL motif. LISP2 is conserved in all *Plasmodium* spp., for which genome sequence data is available. An alignment of LISP2 from five different *Plasmodium* species revealed a conserved PEXEL-like sequence RxLxE (Figure 4D) located approximately 370 amino acids from the N-terminus. Surprisingly, this location is further downstream than in characterised blood stage PEXEL proteins, where the PEXEL position is conserved at 15-40 amino acids from the signal peptide (Boddey *et al.*, 2016; Marti *et al.*, 2004; Sargeant *et al.*, 2006). To assess whether the PEXEL-like sequence in LISP2 is a cleavage site for plasmepsin V, synthetic peptides containing the motif from different *Plasmodium* species were synthesised commercially and incubated with PbPMV-HA agarose (plasmepsin V affinity purified from *P. berghei* parasites using anti-HA agarose) in a PEXEL cleavage assay, as described previously (Boddey *et al.*, 2010; Hodder *et al.*, 2015; Sleebs *et al.*, 2014). Incubation of fluorogenic peptides containing this motif with *P. berghei* plasmepsin V resulted in efficient peptide

cleavage (Figure 4E), whereas incubation of a peptide containing PEXEL RL>A mutations abrogated cleavage. This provides evidence that the PEXEL-like sequence in PbLISP2 can be processed by plasmepsin V. Combined with the lack of LISP2 export in plasmepsin V mutant parasites, this suggests that LISP2 contains an authentic 'PEXEL', despite its recessed location.

Infection with PMVHA-FRT/Flp parasites provides sterile immunity

Parasite arrest during liver stage development can generate varying degrees of protection against future sporozoite challenge. Genetically attenuated parasites (GAPs) targeting UIS3 (Mueller *et al.*, 2005), sap1 (Aly *et al.*, 2011) and p52/p36 (Annoura *et al.*, 2012) have shown promise as whole parasite vaccines, as these preclinical experiments have led to mice developing immunity to sporozoite challenge. As the PMVHA-FRT/Flp parasites arrest during liver stages of development, we examined whether infection with these parasites was able to generate protection against a lethal WT sporozoite challenge. Swiss mice were immunised with up to 3 doses (3-4 weeks apart) of 10,000 PMVHA-FRT/Flp parasites prior to challenge with WT ANKA *P. berghei* sporozoites (Figure 5A). An immunisation dose-dependent increase in sterile protection was observed in immunised mice (Figure 5). Three out of six mice immunised with one dose of PMVHA-FRT/Flp parasites achieved sterile protection and this increased to 9/11 and 25/27 with two and three immunizations with PMV-Flp parasites, respectively (Figure 5). These results confirm that PMV-FLP parasites arrest and are detected by the immune system, allowing immune responses to be generated that protect against lethal challenge.

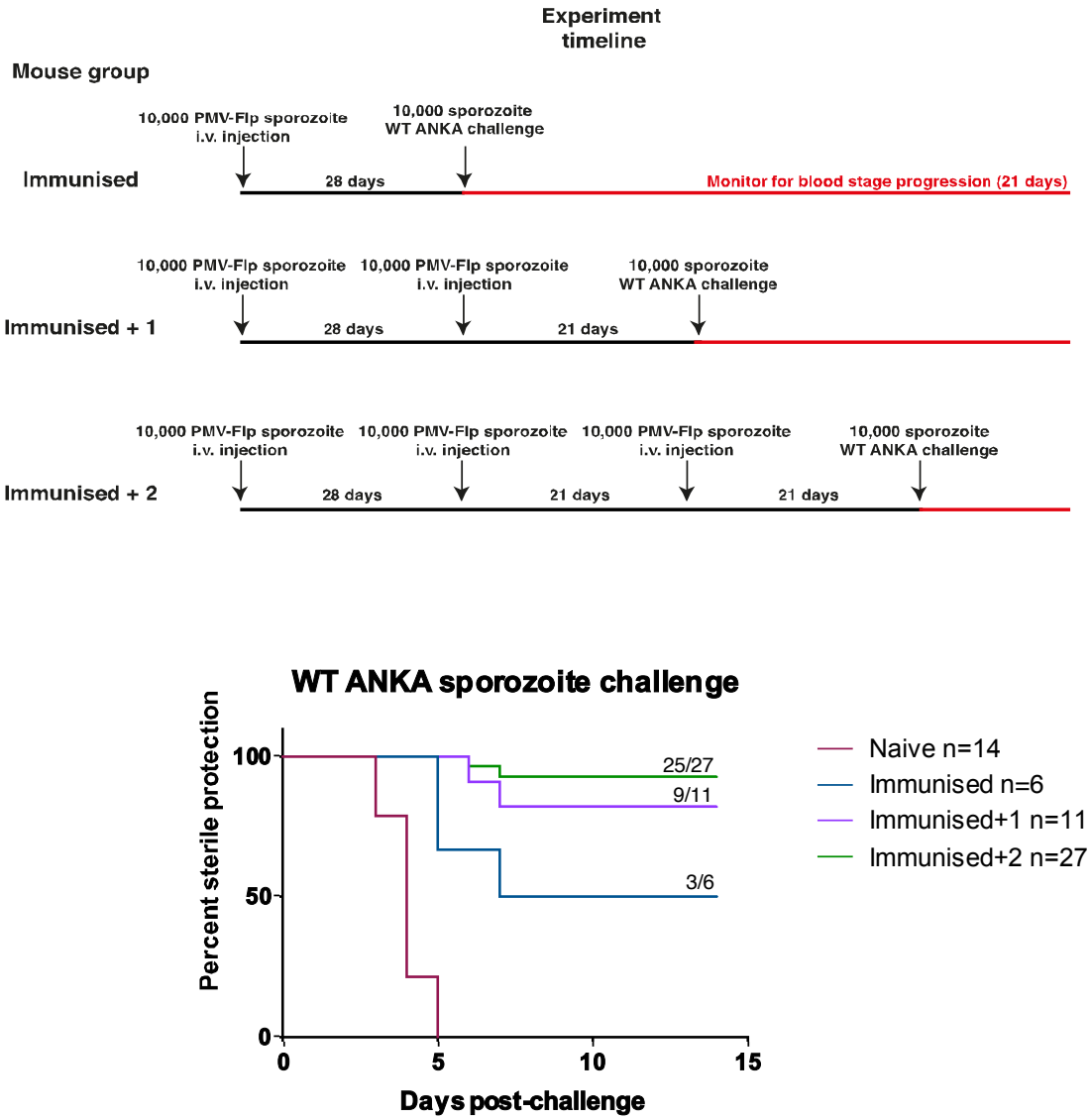


Figure 5: Immunisation study performed using the PMVHA-FRT/Flp parasites as a means of generating protection from sporozoite challenge. Swiss mice were infected with differing number of doses of 10,000 PMVHA-FRT/Flp parasites within 3 different groups (as shown in the timeline above). Mice were then challenged with 10,000 WT ANKA sporozoites and were monitored for blood stage progression for up to 21 days post infection. Naïve (non-immunised) mice were used as a control to show the infectivity of the WT ANKA parasites used for the challenge. The experiment was performed asynchronously for all the groups and results were collated and represented on the graph.

Discussion

This chapter assesses the importance of the aspartyl protease plasmepsin V in mediating protein export in blood and liver stages of infection in the rodent malaria parasite, *Plasmodium berghei*. The disruption of *P. berghei plasmepsin V* achieved through the use of the Flp/FRT 3'UTR excision system (Lacroix *et al.*, 2011) is the first documented example of a conditional knockout of *plasmepsin V* locus. Prior studies have been unable to genetically disrupt the *plasmepsin V* gene in *P. falciparum* or *P. berghei* (Boddey *et al.*, 2010; Klemba & Goldberg, 2005; Russo *et al.*, 2010; Sleebs *et al.*, 2014) due to the presumed essentiality of plasmepsin V. Indeed, development of plasmepsin V specific inhibitors has confirmed that plasmepsin V is essential for parasite survival inside the erythrocyte due to its role in protein export (Gambini *et al.*, 2015; Hodder *et al.*, 2015; Sleebs *et al.*, 2014). Conditional knockdown of plasmepsin V has been achieved in *P. falciparum* (Sleebs *et al.*, 2014) using the *glmS* riboswitch system (Prommana *et al.*, 2013). This resulted in ~90% knockdown of protein levels in asexual parasites but this was insufficient to affect parasite growth due to the potent activity of the remaining pool of enzyme (Sleebs *et al.*, 2014). Plasmepsin V knockdown did however sensitize parasites to plasmepsin V inhibitors, demonstrating that it is indeed a direct target of the compounds (Sleebs *et al.*, 2014).

In the current study, genetic deletion of the *plasmepsin V* locus was achieved at the 3' UTR exclusively within sporozoites developing within mosquitoes. This approach allowed plasmepsin V expression to be retained in asexual stages and gametocytes, when plasmepsin V is essential. We did not detect any significant reduction in salivary gland sporozoite numbers (data not shown), suggesting that plasmepsin V is not essential in mosquito stages of the parasite lifecycle. This is not especially surprising as plasmepsin V functions to export PEXEL proteins beyond the parasitophorous vacuole membrane and mosquito stages are not known to produce this structure in the mosquito. Despite data showing expression of some genes that encode PEXEL proteins, such as *CSP* and *TRSP* that are important for sporozoite development (Coppi *et al.*, 2011; Coppi *et al.*, 2005)

and hepatocyte invasion (Labaied *et al.*, 2007), respectively, our PMVHA-FRT/Flp mutant data suggests that the PEXEL is not important during sporozoite developmental stages. Interestingly, during sporozoite infection of mice, there was no difference in parasite liver load between wildtype and PMVHA-FRT/Flp mutants 6 hours post-infection, suggesting that plasmepsin V does not play a critical role in sporozoite motility or infectivity for liver cells *in vivo*. However, liver-stage arrest was observed at later timepoints, consistent with plasmepsin V being required for development of exoerythrocytic forms inside hepatocytes. This suggests that plasmepsin V is important for export of proteins during hepatocyte infection.

The notion of PEXEL-mediated protein export during liver stages has been explored previously (Cockburn *et al.*, 2011; Ingmundson *et al.*, 2012; Singh *et al.*, 2007), however, the mechanism behind export of CSP and whether PEXEL cleavage directly leads to its export has not yet been determined. Difficulties in studying liver stage protein export arose from the lack of effective immunodetection tools for small amounts of protein material within the host cell. Anti-CSP antibodies have been shown to detect CSP in infected liver cells (Singh *et al.*, 2007), however, due to the fact that CSP is shed during sporozoite traversal of hepatocytes, it is possible that intracellular CSP could be deposited during migration (Cockburn *et al.*, 2011), this data is not enough to confirm the presence of PEXEL-mediated export in liver cells. Since plasmepsin V functions by cleaving the PEXEL motif during export in asexual stages, the PMVHA-FRT/Flp mutant is an important tool to investigate the role of PEXEL-dependent export in the liver stage. The lack of a defect in sporozoite infectivity in mice, but arrest of PMVHA-FRT/Flp parasites between 6-24 h post infection (Figure 3C) suggests that plasmepsin V is required for parasite development early following invasion of hepatocytes, as would be expected if plasmepsin V had a role in protein export. This, together with the fact that plasmepsin V exhibits a highly specific activity for processing the PEXEL motif (Figure 2D, 4E), raises the possibility that PEXEL processing may be occurring during liver stages of infection. However, since PEXEL processing in infected cells is normally measured by a combination of immunoblotting and mass spectrometry (Boddey

et al., 2010; Boddey *et al.*, 2009), it is very difficult to confirm this in liver stages because the efficiency of hepatocyte infection is low, limiting the amount of material that can be prepared. This has been a problem in our and other laboratories. Nonetheless, employing a biochemical PEXEL cleavage assay with plasmepsin V isolated from *P. berghei*, it was possible to demonstrate that the enzyme cleaves the PEXEL sequences present in LISP2 from different *Plasmodium* spp. This is consistent with LISP2 having an authentic plasmepsin V cleavage site required for export to the hepatocyte.

This above data complements the findings of Kalanon *et al.* (2015), where a similar Flp-FRT conditional deletion was employed to knockdown the PTEX translocon component EXP2, showing its importance during liver infection. Additionally, that study showed that the PTEX unfoldase, HSP101, was not detected using immunofluorescence microscopy in *P. berghei*-infected hepatocytes, indicating that the protein export mechanisms in liver stages are similar but may not be identical to that which has been confirmed in blood stage infection (Beck *et al.*, 2014; Boddey *et al.*, 2010; Boddey *et al.*, 2013; Boddey *et al.*, 2009; de Koning-Ward *et al.*, 2009; Elsworth *et al.*, 2014). It is important to note that plasmepsin V PEXEL processing is one of the earliest steps in protein export, as the cleavage event occurs almost immediately after protein translation an the endoplasmic reticulum cytoplasmic leaflet (Boddey *et al.*, 2016; Marapana *et al.*, 2018; Sleebs *et al.*, 2014). It is entirely plausible that the cleavage of the PEXEL motif itself is conserved across both blood and liver stage infection, but the downstream trafficking events, including the interactions with one or more members of the PTEX complex, are what differ between these stages.

This study investigated LISP2, a known liver stage exported protein (Orito *et al.*, 2013), to characterize the protein trafficking phenotype of the PMVHA-FRT/Flp mutant. Using the LISP2 antibody published previously by Orito *et al.* (2013), we showed that PMVHA-FRT/Flp parasites are defective in exporting LISP2 in HepG2 cells (Figure 4). *In vitro* analysis using HepG2 cells was preferentially used over *in vivo* experiments due to the parasites inability to survive to late enough time points where LISP2 expression occurs in infected mice. LISP2

appears to localise to large internal structures surrounding the multiple parasite nuclei during schizogony. Kaiser *et al.* (2016) have reported using high resolution microscopy the appearance of these structures in *P. berghei* liver stages as the parasite's interconnected multiple ER. Whist LISP2 was localized within these structures in HepG2 cells infected with wild-type parasites, it also localised at the parasitophorous vacuole surrounding the exo-erythrocytic form (EEF) and also abundantly within the hepatocyte cytoplasm. In contrast, LISP2 localisation was restricted to the ER in the majority of PMVHA-FRT/Flp-infected cells and was not apparent in either the parasitophorous vacuole and/or host cell. These findings suggest that LISP2 is unable to traffic beyond the ER of the parasite when plasmepsin V is absent. This finding mirrors what is seen during erythrocyte infection when PEXEL mutants are trapped within the endoplasmic reticulum, as they are not processed by plasmepsin V and cannot traffic correctly (Boddey *et al.*, 2010; Boddey *et al.*, 2013). LISP2 does possess a PEXEL motif (RILAE) but it is not found at the canonical location near the N-terminus, but is rather located 373 amino acids away from the start methionine (Orito *et al.*, 2013). As mentioned above, it has been technically very difficult to confirm cleavage at this position by plasmepsin V in EEFs. However, incubation of this sequence within a synthetic peptide substrate with purified *P. berghei* plasmepsin V showed that the sequence is cleaved in a PEXEL-dependent manner, such that RLE>A mutations blocked cleavage, consistent with the cleavage specificity of plasmepsin V (Boddey 2010; Russo 2010). This provides evidence that LISP2 is a substrate of PMV. Intriguingly, the PEXEL-like motif (TEXEL) found in *Toxoplasma gondii* can also be found in atypical locations either in the middle or towards the C-terminus (Coffey *et al.*, 2015; Naor *et al.*, 2018). Collectively, these findings identify LISP2 as being the first exclusive liver-stage PEXEL protein whose export to the hepatocyte is dependent on plasmepsin V.

The PMVHA-FRT/Flp mutant has not only provided the opportunity to examine the protein export mechanism in liver stages, but it has allowed us to also investigate protective immunity to parasites following their arrest during liver infection. Previous genetically attenuated parasites (GAPs) have shown similar

or better levels of protection when compared with the PMVHA-FRT/Flp mutant. UIS3 and UIS4 knockouts can generate complete protection within mice following sporozoite injection (Mueller *et al.*, 2005), however differences in levels of protection are observed when parasites arrest under different circumstances. Sap1 knockout parasites arrest early during liver stages (Aly *et al.*, 2011), prior to vacuole formation. Early arrest is synonymous with lesser protection, as the host is exposed to the parasites for a shorter time than late arresting parasites. Studies also show the effectiveness of GAPs can vary significantly dependent upon the strain of mouse used, the time between injections and the number of challenges given (Annoura *et al.*, 2012; Mueller *et al.*, 2005). The majority of PMVHA-FRT/Flp mutant parasites arrested by 24 hours post-infection; however, some parasites still remain at the 44 hour time-point (approximately 30-fold more than at 6 hours (Figure 3)). It is not known whether parasites arresting by 24 hours, or those arresting later, are providing the protection, but it is likely that later-arresting parasites will provide stronger protection and likely contribute in our experiments. The conditional deletion of plasmepsin V was sufficient to elicit arrest of liver stages, validating the importance of plasmepsin V during liver infection. Perhaps a conditional *P. falciparum* plasmepsin V mutant that arrests in liver stages is worth considering for future GAP studies.

Chapter 4: Characterisation of the exported protein PBANKA_0700700 across the *Plasmodium* lifecycle

Introduction

After the malaria parasite invades a red blood cell, it must extensively remodel the host cell through the export of many effector proteins. This process of protein export often occurs with proteins that featured a highly conserved N-terminal motif, termed the *Plasmodium* export element (PEXEL) (Hiller *et al.*, 2004; Marti *et al.*, 2004). This motif consists of 5 amino acid residues, RxLxE/Q/D, which are recognised and cleaved by the ER-resident aspartyl protease, plasmepsin V (Boddey *et al.*, 2010; Russo *et al.*, 2010). Cleavage occurs after the conserved leucine residue, revealing a new N-terminus, which is acetylated (Boddey *et al.*, 2009; Chang *et al.*, 2008). Trafficking out of the parasite leads proteins to the parasitophorous vacuole and translocation through the *Plasmodium* translocon of exported proteins (PTEX) (Beck *et al.*, 2014; de Koning-Ward *et al.*, 2009; Elsworth *et al.*, 2014) into the host cell in an ATP-dependent manner.

Our understanding of PEXEL-dependent export during the asexual blood stage currently stems from a multitude of studies using *P. falciparum* as a model. There have been far fewer studies investigating the mechanisms of protein export in other species of *Plasmodium*, including the rodent malaria model organism, *P. berghei*. It has been shown that the PEXEL/HT motif is functional in *P. berghei*; the motif can direct a *P. falciparum* PEXEL-GFP fusion protein into the mouse erythrocyte (MacKenzie *et al.*, 2008). Furthermore, transgenic *P. berghei* parasites expressing the N-terminus of the *P. falciparum* exported protein Knob-associated histidine-rich protein (KAHRP), which is absent from the *P. berghei* genome, also exported this reporter beyond the parasitophorous vacuole (Sijwali & Rosenthal, 2010). Interestingly, N-terminal fusions of the major virulence factor *P. falciparum* erythrocyte membrane protein 1(PfEMP1) were not

exported to the *P. berghei*-infected erythrocyte (Sijwali & Rosenthal, 2010). This is likely due to *P. berghei* lacking many of the proteins required for PfEMP1 trafficking in *P. falciparum*. In addition to this evidence, proteomic and genomic analysis has revealed many PEXEL and PNEP candidates suggesting that protein export may indeed be crucial for erythrocytic *P. berghei infection* (Pasini *et al.*, 2013).

P. berghei erythrocytic remodelling has also described for the SEP (small exported protein) family of proteins (Currà *et al.*, 2012) and characterisation of a *P. berghei* exported effector protein, *P. berghei* Cleft-like protein 1 (PbClp1), showed that the PEXEL motif RxLxY was functional in trafficking PbClp1 to discrete punctate structures in the red blood cell (Haase *et al.*, 2013). Schizont membrane-associated cytoadherence protein (SMAC), a *P. berghei* protein important for erythrocytic sequestration, also contains a PEXEL and is exported to the host cytoplasm (Fonager *et al.*, 2012). Proteins of the pir, fam-a and fam-b multigene families were also reported as exported proteins in *P. berghei* blood stages, however, these mCherry-tagged proteins only localised to the parasitophorous vacuole during the liver stage (Fougère *et al.*, 2016), suggesting either that they are not exported proteins in liver stages or that the mCherry tag interferes with translocation into hepatocytes. Additionally, the *P. berghei* induced structures protein 1 (IBIS1) is another exported protein in both blood and liver stages of infection and its localisation and function appear to be similar to that of *P. falciparum's* Maurer's clefts (Ingmundson *et al.*, 2012). Together, these studies provide evidence for a functional PEXEL motif and protein export in *P. berghei* blood stages.

Some members of PTEX have also been investigated in *P. berghei*, with the consensus being that this complex is required for export (Elsworth *et al.*, 2014; Kalanon *et al.*, 2015; Matz, Goosmann*, et al.*, 2015; Matz, Ingmundson*, et al.*, 2015; Matz *et al.*, 2013). Interestingly, the HSP101 unfoldase component may be absent from *P. berghei* liver stages (Kalanon *et al.*, 2015). Whether this is also the case in *P. falciparum* liver stages remains unknown.

The specific function of plasmepsin V and whether the PEXEL motif is cleaved is in *P. berghei* during export is still unknown. In this study, we investigated a previously uncharacterised protein, PBANKA_0700700, which contains a putative PEXEL sequence, suggesting it is exported. The PBANKA_0700700 gene is transcribed in blood and liver stages of the lifecycle, providing an ideal opportunity to examine the mechanism of protein export in both stages and study the function of the protein across the lifecycle.

PBANKA_0700700 constructs

To study the function of the *P. berghei* gene *PBANKA_0700700*, it was disrupted using a double crossover strategy based on homologous recombination of targeting constructs into the genome of the parasite (Figure 1A, KO was performed in the laboratory of Dr. Blandine Franke-Fayard). Targeting sequences were amplified using *P. berghei* ANKA genomic DNA using primers specific for the 5' and 3' UTR regions of PBANKA_0700700. These regions were cloned into the pL0001 plasmid (MR4), flanking the pyrimethamine resistant *Toxoplasma gondii* (*Tg*) dihydrofolate reductase-thymidylate synthase (DHFR) as a selectable marker under control of the *P. berghei dhfr/ts* promoter.

To study the role of the PEXEL motif in trafficking and function of PBANKA_0700700, transgenic parasites were generated by replacement of the endogenous gene with a 3xHA-tagged copy along with a 3xHA-tagged PEXEL mutant copy, where the RLS residues of the PEXEL were mutated to alanines (Figure 6B, 7A) using double crossover homologous recombination with the Pb3 plasmid, modified with a human DHFR resistance cassette. Additionally, an m-Cherry tag was introduced into the endogenous PBANKA_0700700 locus at the C-terminus using similar crossover strategies in order to investigate protein trafficking (Figure 1C, Blandine Franke-Fayard, unpublished). Clonal lines were generated through limiting dilution of the parasites in mice.

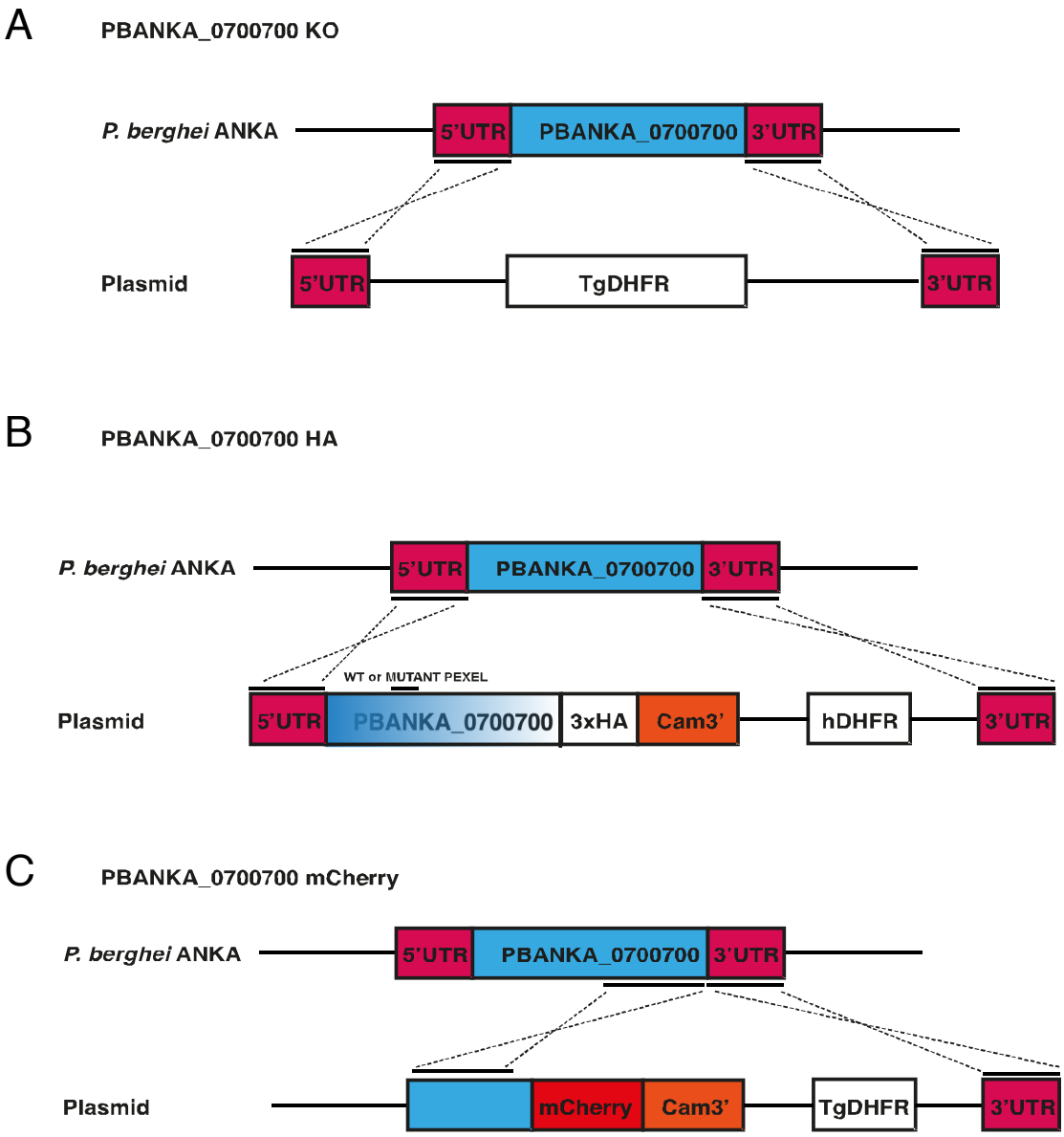


Figure 6: A. Plasmid schematic showing the genetic deletion of endogenous PBANKA_0700700 and its replacement with *TgDHFR*.
B. Plasmid schematic showing the replacement of the endogenous PBANKA_0700700 gene with a codon optimised version featuring either the WT PEXEL motif or a mutated (RLS>A) motif. **C.** Plasmid schematic showing the incorporation of the mCherry tag to PBANKA_0700700.

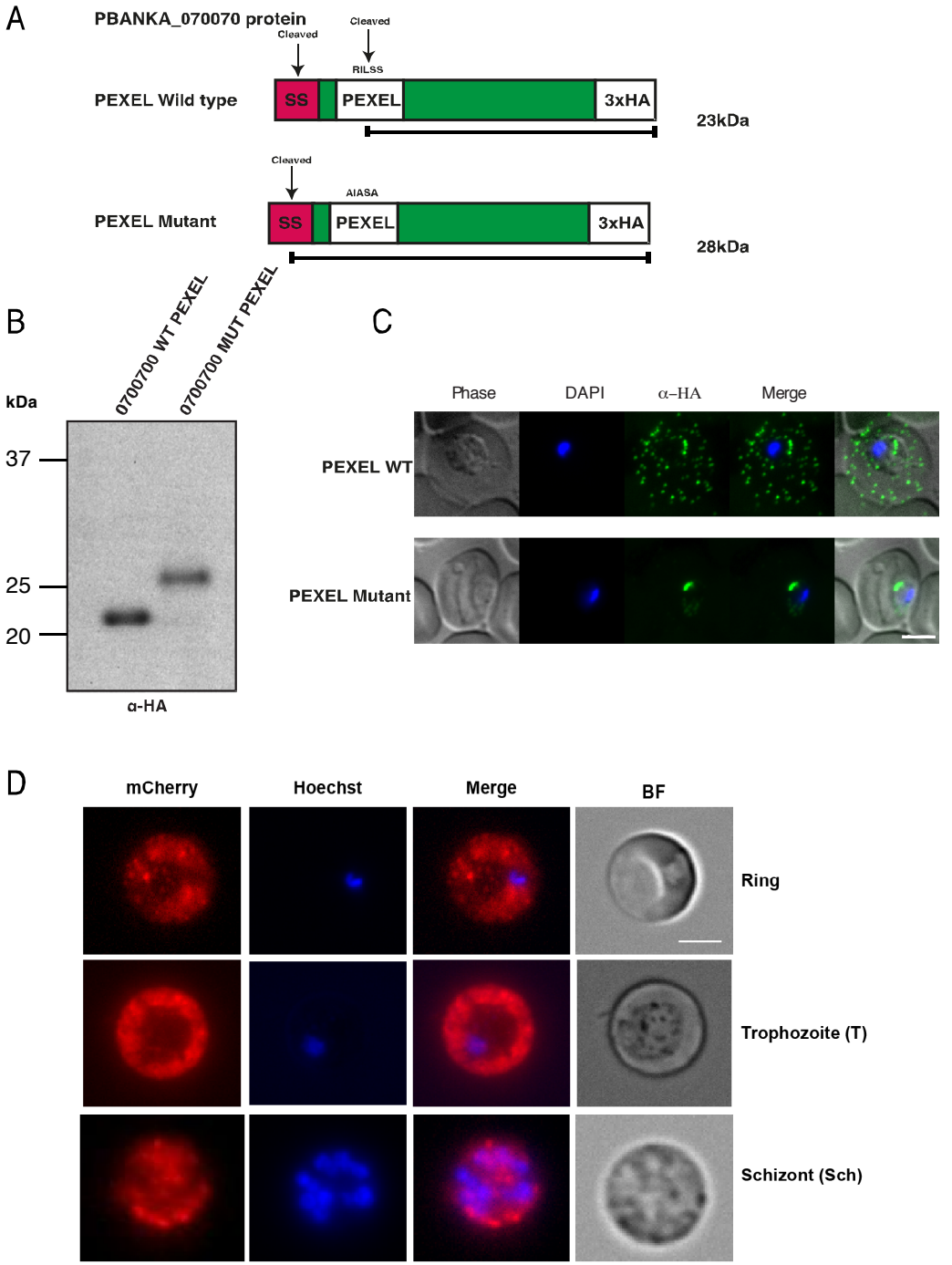


Figure 7: A. Diagram revealing the predicted cleavage sites for both the 07 WT and 07 MUT PEXEL proteins. Expected sizes of the resulting protein are also indicated. **B.** Western blot analysis of saponin treated blood stage parasite material for both 07 WT and 07 MUT lines. Cleavage of the PEXEL motif results in a 23 kDa band for 07 WT, however, the PEXEL mutant band runs at 28 kDa suggesting the PEXEL was not cleaved. **C.** Immunofluorescence analysis of ring stage *P. berghei* parasites from mouse erythrocytes. The green staining indicates the localisation of 0700700 to structures within the erythrocyte cytoplasm for the WT. The 07 MUT PEXEL was confined to the parasite and was not exported. Scale = 2um **D.** Live imaging of blood stage parasites expressing PBANKA_0700700 tagged with mCherry. Image provided by Dr Blandine Franke-Fayard.

PBANKA_0700700 contains a PEXEL motif required for export that is cleaved by plasmepsin V during blood stage infection.

PBANKA_0700700 contains a predicted N-terminal signal peptide for entry into the parasite secretory pathway and a putative PEXEL sequence, RILSS (Figure 7A). To investigate the function of the PEXEL-like sequence and the localization of PBANKA_0700700, we generated transgenic *P. berghei* parasites in which the endogenous gene was tagged at the C-terminus with 3x HA tags by double cross-over homologous recombination (Figure 6B). One parasite line retained the native PEXEL sequence whilst a control parasite line was generated in which the PEXEL sequence was mutated RLS>A (Figure 6B). To confirm that parasites were expressing the tagged protein, saponin pellets of infected mouse blood samples were prepared and analysed by immunoblotting using anti-HA antibodies. Expression of a HA-specific band was apparent in both the WT and PEXEL mutant lines, however the size of the band in PEXEL mutant parasites was larger than in WT parasites (Figure 7B). The predicted molecular weight of full-length or signal peptide-cleaved PBANKA_0700700-HA is 28 kDa, consistent with the band observed in the PEXEL mutant (Figure 7B). The smaller HA species present in WT parasites indicates N-terminal processing of the protein occurred and this required the conserved PEXEL residues, consistent with cleavage by plasmepsin V. The observed size of ~23 kDa is as expected following processing after leucine within the PEXEL motif.

To investigate the function of the PEXEL sequence and proteolytic processing on trafficking of PBANKA_0700700 in infected erythrocytes, immunofluorescence microscopy of the transgenic parasite lines was performed. Anti-HA immunostaining of the WT PEXEL transgenic line detected the protein inside the parasite within a bright structure close to the nucleus, suggestive of the ER, and in discrete punctate structures within the host erythrocyte, confirming it is exported (Figure 7C). This localization is similar to IBIS1 suggesting it is membrane-associated (Ingmundson *et al.*, 2012; Petersen *et al.*, 2015). This localization was surprising in that PBANKA_0700700 does not possess a

predicted transmembrane domain, suggesting that it is peripherally associated with membranes in the host cell via interactions with other proteins, as has been reported for several *P. falciparum* exported proteins at the Maurer's clefts and J-dots (Boddey & Cowman, 2013). C-terminal tagging with mCherry confirmed that PBANKA_0700700 is exported to the host erythrocyte and localises in puncta (Figure 7D). Conversely, mutation of the PEXEL RLS>A inhibited export of PBANAKA_0700700, with the mutant protein accumulating inside the parasite within a bright structure close to the nucleus, again suggestive of the ER (Figure 7C). This localization in the parasite ER is consistent with inhibited PEXEL cleavage by PMV as a result of mutating the PEXEL sequence, causing accumulation within this organelle, as is the cause for PEXEL mutant proteins in *P. falciparum* that are not cleaved by PMV (Boddey *et al.*, 2010; Boddey *et al.*, 2013; Boddey *et al.*, 2009; Sleebs *et al.*, 2014). Altogether, these results provide the first example of plasmepsin V-dependent PEXEL cleavage and protein export in *P. berghei*-infected erythrocytes. This shows that the export mechanism for PEXEL proteins is conserved between different *Plasmodium* spp., as has been hypothesized previously (Marti *et al.*, 2004; Sargeant *et al.*, 2006; Sleebs *et al.*, 2014).

PBANKA_0700700 is expressed during liver stages and may be exported to the hepatocyte nucleus in a PEXEL-dependent manner

Having determined that PBANKA_0700700 is an exported protein in asexual stages, its expression and localisation during liver infection was investigated. HepG2 hepatocytes were infected with transgenic parasites expressing PBANKA_0700700-mCherry and expression and localization was monitored at LUMC. At 30 hours post-infection (hpi), no expression was observed. However, mCherry was detected within the liver schizont from 40 hpi interspersed with the numerous merozoite nuclei and a stronger signal was observed at the parasite periphery, in punctate spots reminiscent of the parasitophorous vacuole

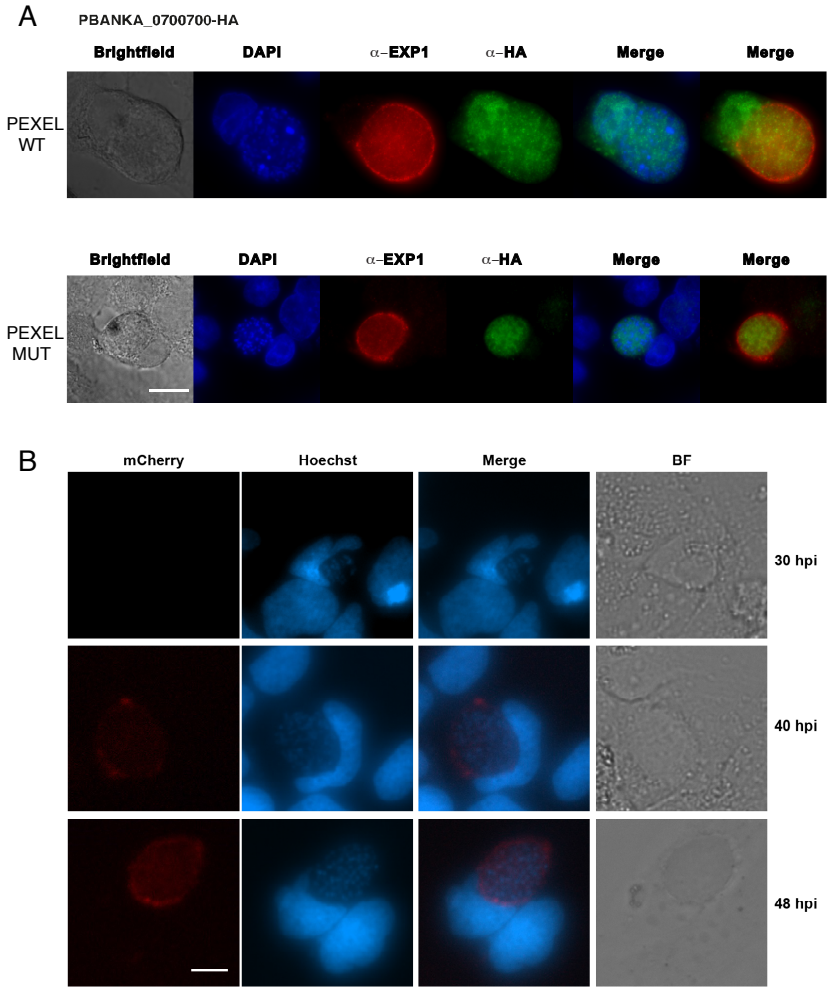


Figure 8: A. Immunofluorescence assay of *P. berghei* infected HepG2 hepatocytes 44 hpi. Staining with anti-EXP1 (red) and anti-HA (green) revealed export of PBANKA_0700700 (WT PEXEL) to the hepatocyte nucleus (scale = 10 μm). The PEXEL mutant was not exported and was contained within the parasitophorous vacuole (red). **B.** Live imaging of infected hepatocytes with 0700700 mCherry. Expression of PBANKA_0700700 appears to begin during the later liver stages starting from 40 hpi. PBANKA_0700700-mCherry localises to the parasitophorous vacuole but does not get exported to the hepatocyte. Image provided by Dr Blandine Franke-Fayard.

(de Koning-Ward *et al.*, 2009; Wickham *et al.*, 2001) (Figure 8B). This suggests that PBANKA_0700700-mCherry was secreted from inside the parasite to the parasitophorous vacuole where it accumulated and was not exported into the hepatocyte. This result is similar to that reported previously, where export of GFP or mCherry-tagged PEXEL reporters occurred in *P. berghei*-infected erythrocytes but export beyond the parasitophorous vacuole was not observed in hepatic stages (Fougère *et al.*, 2016; Ingmundson *et al.*, 2012; Kalanon *et al.*, 2015). It is possible either that PBANKA_0700700 and the previous PEXEL reporters are not exported by hepatic stages, or that export was hampered by the mCherry/GFP tags in liver stages, or that the population of exported proteins is very low.

One possibility we wanted to explore further was if the mCherry tag could block export into the hepatocyte cytosol. This has been reported previously for the PEXEL-containing protein LISP2 (Orito *et al.*, 2013), also called Sequestrin (Annoura *et al.*, 2014), a protein whose export into hepatocytes we show in Chapter 3 is PMV-dependent however, its export is blocked when mCherry is fused to the C-terminus. Translocation of proteins at the PVM in blood stages involves the PTEX translocon, with cargo being unfolded by the PTEX component, Heat Shock Protein 101 (HSP101) (Beck *et al.*, 2014; de Koning-Ward *et al.*, 2009; Elsworth *et al.*, 2014; Kalanon *et al.*, 2015). Kalanon *et al.* (2015) reported that HSP101 expression is absent in *P. berghei* liver stages. This suggests that unfolding of proteins by HSP101 may not occur in hepatic stages, possibly including those which are fused to GFP/mCherry, or that an alternative chaperone is involved in translocation. To investigate this hypothesis, we studied the expression and localisation of PBANKA_0700700 in liver stages using HA-tagged proteins described above (refer to Figure 6) by immunofluorescence microscopy. HepG2 cells were infected with sporozoites and fixed at 48 hours post infection, then consequently stained with anti-HA and anti-EXP1 antibodies, the latter being a marker of the PVM. In some infected cells, HA-specific signal was observed beyond the EXP1-labelled parasitophorous vacuole membrane surrounding the liver stage parasite, with the signal co-localizing with the host hepatocyte nucleus (Figure 8A). To determine whether this trafficking involved

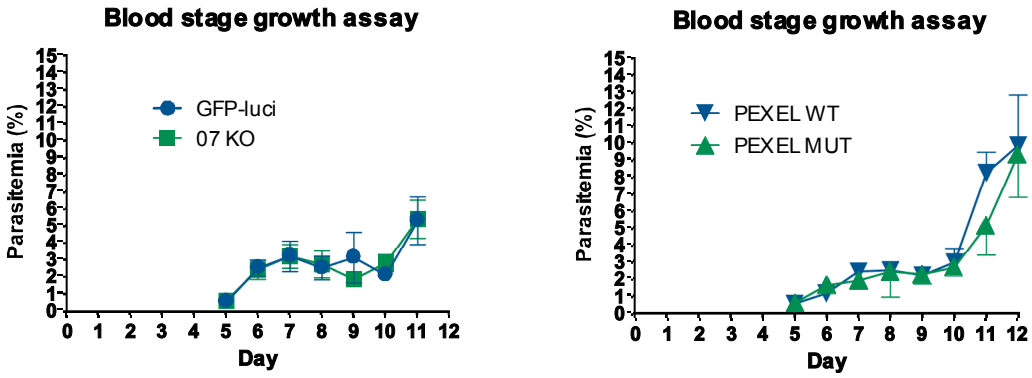
the PEXEL motif, HepG2 cells were infected with the PEXEL mutant PBANKA_0700700-HA parasite line. In all infected cells, the HA-specific signal localised at or within the EXP1-labelled PVM, indicating the protein was not exported but accumulated inside the parasite or PV. These data suggest that PBANKA_0700700 is an exported liver stage protein that requires a functional PEXEL motif to be trafficked into the hepatocyte. Similarly to LISP2, its translocation across the PVM is blocked by mCherry/GFP tags. Inspection of PBANKA_0700700 identified the presence of several nuclear localisation sequences, providing a mechanism for the protein to be transported there following translocation into the hepatocyte cytoplasm.

PBANKA_0700700 is dispensable in blood and liver stages

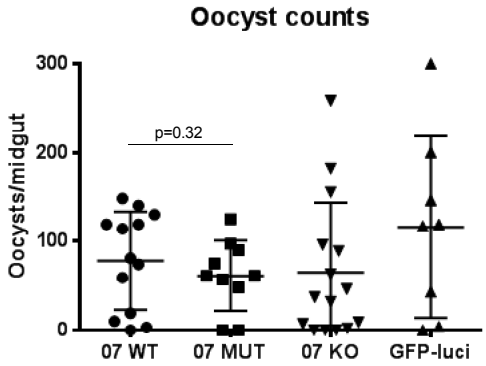
To analyse the function of PBANKA_0700700, the coding sequence was disrupted in the genome of *P. berghei* ANKA by double cross-over homologous recombination (Figure 6A). Individual mutant clones were prepared by limiting dilution in mice and the genotypes were confirmed. These experiments were performed in the laboratory of Blandine Franke-Fayard and Chris Janse (Leiden University Medical Centre), who generously provided the knockouts for functional evaluation.

The essentiality of PBANKA_0700700 and also its PEXEL motif during the asexual blood stage was investigated in parasite growth assays. Mice were intravenously injected with 1000 blood stage parasites and subsequent parasitemia was measured daily from day 3 post-infection. No differences in the parasite growth rate was observed for either the 07 KO compared to the GFP-luciferase expressing control, or the PBANKA_0700700 with wildtype PEXEL (07 WT) versus the RLS>A PEXEL mutant (07 MUT) (Figure 9A). All parasites developed normally throughout blood stages and showed no visible abnormalities (results not shown). This data altogether suggests that *PBANKA_0700700* is not an essential gene during the asexual erythrocytic cycle.

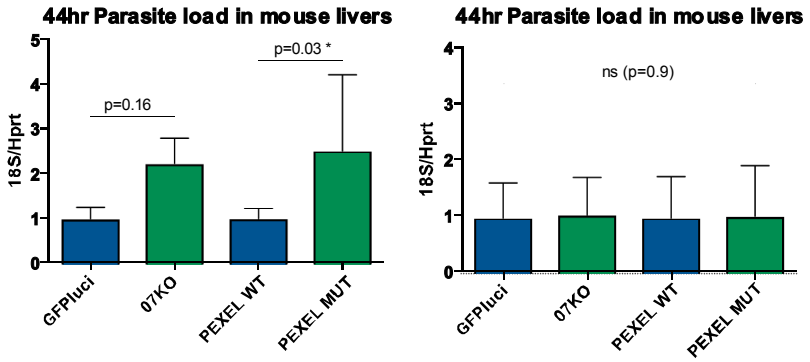
A


Blood stage growth assay
Parasitemia (%)
Day
GFP-luci
07 KO
Blood stage growth assay
Parasitemia (%)
Day
PEXEL WT
PEXEL MUT

B


Oocyst counts
Oocysts/midgut
p=0.32
07 WT
07 MUT
07 KO
GFP-luci

C


44hr Parasite load in mouse livers
18S/Hprt
p=0.16
p=0.03 *
GFPluci
07KO
PEXEL WT
PEXEL MUT
44hr Parasite load in mouse livers
18S/Hprt
ns (p=0.9)
GFPluci
07KO
PEXEL WT
PEXEL MUT

D

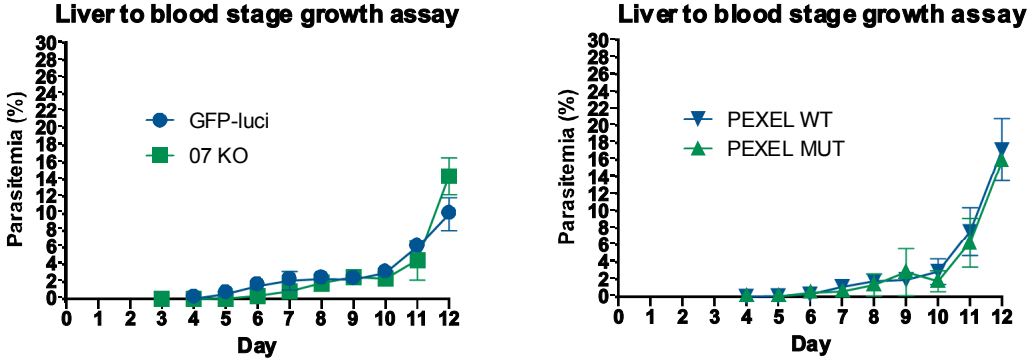

Liver to blood stage growth assay
Parasitemia (%)
Day
GFP-luci
07 KO
Liver to blood stage growth assay
Parasitemia (%)
Day
PEXEL WT
PEXEL MUT

Figure 9: A. Parasitemia counts from mice infected with 1000 blood stage parasites from any of the 4 examined transgenic lines. No significant difference in parasitemia was observed across time for any of the lines. One representative data set is shown from n=3. **B.** Oocyst counts from infected mosquito midguts appeared to be consistent between the PEXEL WT/MUT and KO/luciferase parasite lines. Sporozoite numbers varied between 5000-8000 per mosquito for all lines and was not significantly different (data not shown). **C.** Two independent experiments of quantitative real-time PCR analysis of relative liver parasite load at 44hr in mice infected with 10,000 sporozoites. No consistent significant difference between parasite load was observed across all parasite lines, suggesting no critical function for PBANKA_0700700 during the liver stage. **D.** Blood stage parasitemia measurements taken from mice (n=6) infected with 10,000 sporozoites. Parasitemia did not vary between lines indicating no growth defect apparent for either the 07 KO or 07 PEXEL mutant lines, when compared with the control. One representative data set is shown from n=3.

To investigate the possibility the PBANKA_0700700 has an important function elsewhere in the lifecycle, parasites were differentiated into gametocytes and transmitted to *Anopheles stephensi* mosquitoes by direct mosquito bite in the WEHI insectary. There was no significant difference between oocyst or sporozoite numbers across all examined transgenic lines (Figure 9B), indicating a lack of an essential role during mosquito stages of infection.

Next, we sought to study whether PBANKA_0700700 is important during the liver stage of the lifecycle. Sporozoites dissected from mosquito salivary glands were injected intravenously into mice and parasite liver load was measured by quantitative real-time PCR 44 hours post-infection. There was no consistent significant difference between the parasite liver loads of mice injected with either the 07KO, GFP-luciferase ANKA, PBANKA_0700700 WT or MUT PEXEL lines (Figure 9C). The difference observed between the PEXEL WT and mutant could not be replicated in repeated experiments. This indicates that PBANKA_0700700 is not required for sporozoite infectivity or liver-stage development up until the 44 hour time point. To determine whether PBANKA_0700700 plays a role late in the liver stage, mice injected with sporozoites were allowed to establish a patent infection. Parasites from all 4 lines were capable of establishing blood stage infection by day 3-4 across 2 independent experiments, indicating no delay in the liver-to-blood stage transition (Figure 4D). Parasitemia measurements following progression to blood stage also showed no difference in growth rate (Figure 9D). These findings demonstrate that PBANKA_0700700 is not essential for liver-stage development or for egress from hepatocytes to initiate or sustain a patent infection in mice.

Discussion

This work uses PBANKA_0700700 as a tool to study protein export in *P. berghei*, further enhances our understanding of how PEXEL-mediated export occurs across different species and stages of the malaria life cycle. Our prior

understanding of plasmepsin V activity and PEXEL cleavage was limited to blood stage protein export in *P. falciparum* (Boddey *et al.*, 2010; Hiller *et al.*, 2004; Marti *et al.*, 2004) and plasmepsin V activity from using recombinant proteins from *P. vivax* (Sleebs *et al.*, 2014). This dissertation has revealed the first example of PEXEL-dependent cleavage of a *P. berghei* protein and that this cleavage event is essential for protein export to the infected erythrocyte, consistent with processing by plasmepsin V. This supports the data shown in Chapter 3 that *P. berghei* plasmepsin V has the same substrate specificity as *P. falciparum* and *P. vivax* plasmepsin V (Sleebs *et al.*, 2014).

Mechanisms of protein export during liver stage infection are considered controversial in the literature (Cockburn *et al.*, 2011; Kalanon *et al.*, 2015; Orito *et al.*, 2013; Singh *et al.*, 2007). In the previous chapter, plasmepsin V was shown to cleave the PEXEL sequence in LISP2 peptides, and to be essential for export of LISP2 and for parasite survival within hepatocytes *in vivo*, providing strong evidence that the PEXEL motif is indeed functioning in a similar manner for export during both the blood and liver stages. Expression of mCherry and HA-tagged PBANKA_0700700 allowed for analysis of protein export using a tagged protein, unlike CSP (Singh *et al.*, 2007) and LISP2 (Orito *et al.*, 2013) which were both detected using antibodies to the native protein rather than to large tags. Conflicting results were observed, as the mCherry tagged PBANKA_0700700 protein was restricted to within the PVM, whilst the PBANKA_0700700 HA WT PEXEL protein was exported into the host cell nucleus in some cells. This difference in localisation can be attributed to the different tag used. Previous studies utilising GFP and mCherry tags to localize proteins in infected hepatocytes have only been able to detect secretion to the parasitophorous vacuole and never the presence of the protein within the hepatocyte cytoplasm (Fougère *et al.*, 2016; Kalanon *et al.*, 2015) A similar result was seen with LISP2, as the authors showed LISP2 export to the host hepatocyte using an antibody, however, a LISP2 mCherry fusion was trapped within the parasite and was not exported (Orito *et al.*, 2013). The authors did not explore this difference further, but it follows that studying liver stage protein export requires the use of antibodies or smaller less obtrusive or folded tags (such as HA or c-myc), rather

than larger ones, rendering GFP and mCherry fusions less than ideal. The fact the export of mCherry and GFP tagged reporters proceeds in blood stages suggests that differences in the mechanism or machinery exist between blood and liver stages.

It is also hypothesized that HSP101, the PTEX protein involved in unfolding exported proteins prior to translocation, is not present during malarial liver stages (Kalanon *et al.*, 2015), despite being required in blood stages (Beck *et al.*, 2014; Elsworth *et al.*, 2014). This possibly explains the different protein localisation observed with different tags, but also suggests that the protein complex(es) required for export is different in blood and liver stages.

One of the differences between liver cells and erythrocytes is that the liver cells are nucleated. Interestingly, the export of PBANKA_0700700 in liver cells was directed towards the hepatocyte nucleus. PBANKA_0700700 also features a predicted nuclear localisation sequences close to its c-terminus, which may be involved in trafficking to the host cell nucleus. This reveals the potential for malarial liver stage protein export to manipulate host cell transcription using exported transcription factors, which has been reported previously for CSP (Singh *et al.*, 2007). Similarly, the PEXEL-like export system present in *Toxoplasma gondii* also involves proteins exported to the host cell nucleus (Coffey *et al.*, 2015). ASP5 (the PMV homologue in *Toxoplasma*) cleaves a PEXEL like motif in the protein GRA16, initiating its export into the host nucleus (Bougdour *et al.*, 2013; Coffey *et al.*, 2015). Interestingly, the fusion of GFP to the C-terminus of GRA16 prevents its translocation across the parasitophorous vacuole (Coffey *et al.*, 2015), providing further evidence that certain tags may disrupt protein export. The similarities between *Toxoplasma gondii* host cells and hepatocytes may suggest that liver stage protein export may more closely resemble the export processes in *Toxoplasma* rather than blood stage malarial protein export. This explains the disparateness of conclusions reached about liver stage protein export in literature, that is, whether it is occurring (Cockburn *et al.*, 2011; Singh *et al.*, 2007) and whether plasmepsin V and the PTEX components are essential in the liver stage.

Chapter 5: Targeting the host hepatocyte to promote clearance of *Plasmodium* liver infection

Introduction

The generation of the first *plasmepsin V* gene deletion in *Plasmodium* parasites using *flp* recombinase has enabled the production of liver stage parasites that are defective for export of LISP2 and presumably other unidentified proteins. The strong decrease in viability of plasmepsin V mutant liver stages suggests that the protein export pathway is necessary for exoerythrocytic forms to develop to mature hepatic schizonts and transition to blood stages. The questions that immediately become apparent are: what host cell processes are being manipulated by the parasite during liver stage infection, what parasite effector proteins are involved and how does export facilitate these changes? Protein export during blood stage infection is essential for parasite growth as it allows the parasite to remodel the erythrocyte and evade host immune responses (Hodder *et al.*, 2015; Maier *et al.*, 2008; Sleebs *et al.*, 2014). It is so far unclear what essential functions protein export manipulates in hepatocytes but these cells are substantially different to erythrocytes, including the presence of innate immune responses.

Most cells including hepatocytes have evolved innate immune mechanisms to detect invading pathogens and activate cell death pathways to clear the infection. This includes the activation of apoptosis signaling via binding of extracellular ligands such as TNF to their cognate receptor, such as TNFR, on infected cells to signal the cell to die, clearing the pathogen. *Plasmodium* parasites must subvert or inhibit these immune responses to survive and continue their lifecycle. The parasite's ability to suppress host cell apoptosis has been described previously, with *P. berghei* exoerythrocytic forms protecting infected hepatocytes from apoptotic stimuli whilst cell death occurred in uninfected hepatocytes (van de

Sand *et al.*, 2005). Furthermore, previous data has revealed that *P. yoelii*-infected hepatocytes are upregulated for pro-survival proteins such as Bcl-2 and down-regulated for pro-apoptotic proteins such as p53 (Kaushansky, Ye*, et al.*, 2013). Liver stage parasite viability and suppression of apoptosis is completely reliant upon the formation of the parasitophorous vacuole surrounding the developing parasites (Kaushansky, Metzger*, et al.*, 2013; Spielmann *et al.*, 2012; van Dijk *et al.*, 2005). It is possible that export of parasite proteins beyond the parasitophorous vacuole membrane is involved in suppression of host innate immune responses to *Plasmodium* infection, including hepatocyte apoptosis, as is the case for numerous other intracellular parasite, bacterial and viral pathogens. Indeed, the relatively swift death of plasmepsin V mutant parasites following hepatocyte infection may be due to the inability of parasites to prevent host innate responses, work which is ongoing but beyond the scope of this thesis.

However, to investigate the potential importance of apoptotic signaling during *Plasmodium* development in hepatocytes, we employed both genetic and chemical biology approaches. First, mice possessing deletions in the genes encoding cellular inhibitor of apoptosis proteins (cIAPs) were infected with *P. berghei* sporozoites and we assessed whether the parasites could establish a liver infection and, if so, whether they could initiate a patent infection. Knockouts in these genes were chosen because these proteins are a vital checkpoint at which the decision for a cell to survive or die is determined in numerous extrinsic cell death signaling cascades. Second, we used a chemical antagonist of cIAPs to investigate whether treating wildtype mice post-sporozoite challenge could diminish parasite development in the liver and blood through activation of hepatocyte cell death as a potential mechanism to target host hepatocytes to clear *Plasmodium* infections.

Previous studies have shown that p53 agonists and bcl-2 inhibitors promote apoptosis in infected hepatocytes and this was effective in reducing parasite load in both *in vitro* and *in vivo* models (Douglass *et al.*, 2015; Kaushansky, Metzger*, et al.*, 2013). However, while these prior studies focused on the intrinsic pathways of apoptosis, an alternative approach has been to utilize the extrinsic apoptosis signaling pathway to clear hepatitis B virus (HBV) infections in the livers of mice

using SMAC mimetics (Ebert, Allison, *et al.*, 2015; Ebert, Preston, *et al.*, 2015a). SMAC mimetics are compounds that mimic the activity of the native SMAC protein in cells, which regulates cIAP levels via ubiquitination and proteosomal degradation in a death receptor-dependent manner.

In this chapter, the aim was to examine whether hepatocyte cell death signaling pathways are involved in the development of liver stage parasites. The efficacy of a SMAC mimetic was also assayed as an alternative pro-apoptotic therapy to clear *Plasmodium* liver stages. A deeper understanding of the apoptosis pathways during *Plasmodium* liver infection could inform future studies aimed at identifying whether exported proteins are involved in suppressing host cell death and may also pave the way for future therapies that target the host cell to clear *Plasmodium* infections and induce immunity.

cIAP knockout mice control *Plasmodium* liver infection

To determine whether apoptosis signaling is involved in liver infection by malaria parasites, knockout mice lacking cIAP1 (all cells) and cIAP2 (hepatocytes only) were injected with 10,000 *P. berghei* gfp-luciferase sporozoites, alongside wild-type (WT) control C57BL/6 mice and parasite liver load was quantified by qPCR at different times. At 6h post infection (Figure 10A), parasite load did not vary between cIAP1/2 KO mice and C57BL/6 controls, suggesting sporozoites were able to migrate to the liver and infect hepatocytes as expected. However, at 20h and 44h post-infection, the parasite load in cIAP1/2 KO mice was significantly lower than in C57BL/6 controls.

Blood prepared from the infected mice was analysed for alanine aminotransferase (ALT), an enzyme only present in blood following liver cell damage or death. Elevated levels were observed in cIAP1/2KO mice at both the 20h and 44h timepoints (Figure 10B), suggesting that the reduction of parasite load is linked to liver cell death presumably due to increased apoptotic signaling in the absence of cIAP1 and cIAP2 in the liver. One mechanism through which this could occur is TNF-dependent apoptosis (Ebert, Allison, *et al.*, 2015; Ebert, Preston, *et al.*, 2015a). Indeed, immunoassay analysis of serum TNF-α levels

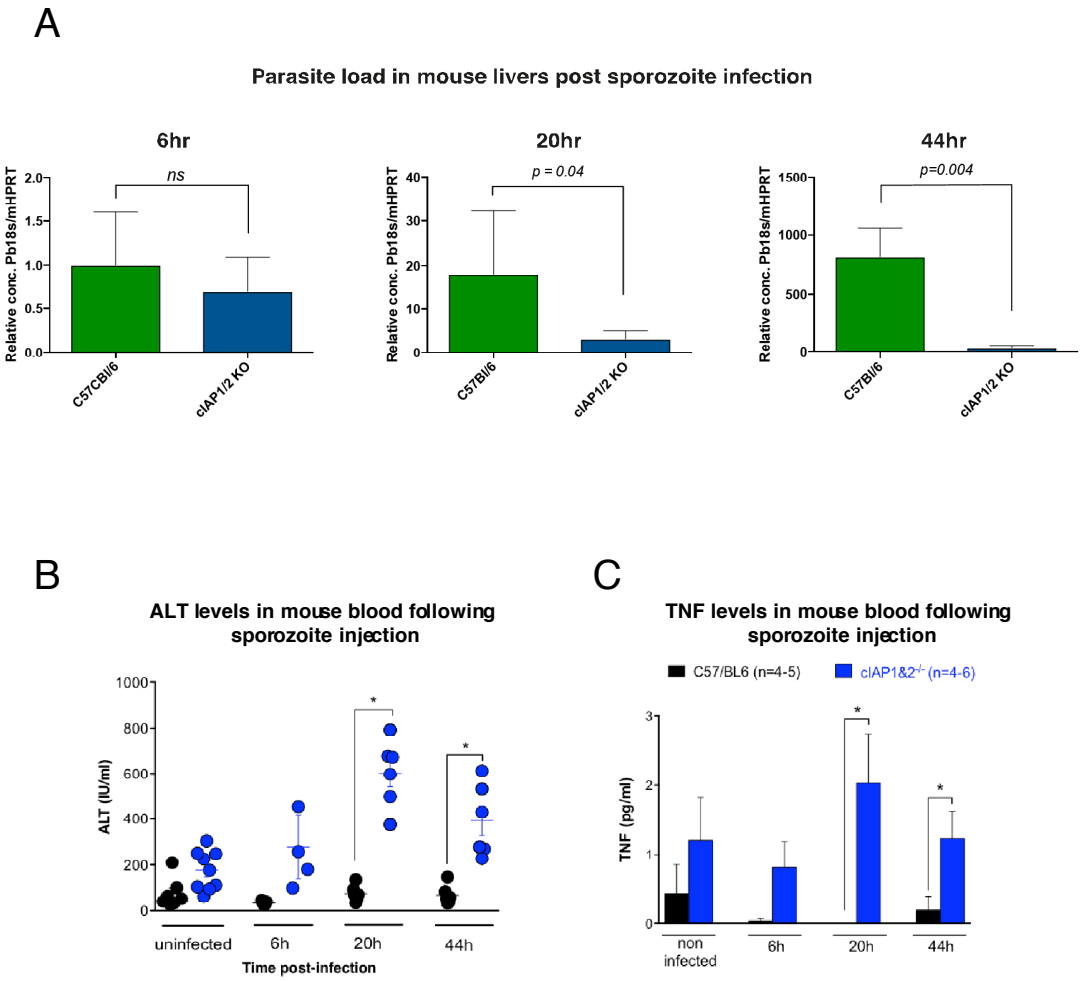


Figure 10: A. Quantification of liver parasite load using RT-PCR in WT and cIAP1/2 KO mouse livers at different time points (6h, 24h and 44h) following 10,000 sporozoite intravenous injection. **B.** Levels of alanine aminotransferase (ALT) in serum of uninfected and *P. berghei* infected C57Bl/6 mice. Data provided by Dr. Greg Ebert. **C.** TNFα levels in mouse serum post *P. berghei* infection measured using an immunoassay.

showed higher levels in cIAP1/2KO mice at the 20h and 44h time points (Figure 10C), similar to the time points at which ALT was increased (Figure 10B). Interestingly, higher TNF-α levels were previously reported during HBV infection and this was exploited using a SMAC mimetic called birinapant to clear HBV infection (Ebert, Preston*, et al.*, 2015a). The similarity of these mechanisms suggested that birinapant may also be efficacious in clearing *Plasmodium* liver infections.

A SMAC mimetic reduces *Plasmodium* liver infection

One key difference between HBV and *Plasmodium* liver infections is that HBV causes a chronic liver stage illness that can be cleared over many months with continuous treatment of the SMAC mimetic, birinapant. Conversely, *Plasmodium* parasites infect the liver for a short period of time (2-3 days for *P. berghei*), which makes the window of drug administration much narrower. To test the efficacy of birinapant in the context of malaria, groups of mice were administered the compound (40 mg/kg) or DMSO vehicle control by i.p. injection 6 hours prior to sporozoite challenge to allow cIAP1/2 degradation. Mice were then injected with 10,000 *P. berghei* sporozoites i.v. and parasite liver load measured 6 hours post infection by qPCR (Figure 11A). No significant difference was observed between the birinapant and vehicle treatments as expected based on the previous results with the cIAP1/2 KO mice (Figure 10A). This demonstrates that birinapant does not inhibit the ability of sporozoitees migrating to or infecting the liver in mice.

Next, mice were treated as above and parasite liver measured 44h post infection. The birinapant treatment significantly reduced the parasite burden when compared to the vehicle (Figure 11B), suggesting birinapant is indeed effective at killing parasites, or their host cells, during liver stage development. However, birinapant treatment did not results in complete parasite clearance. Further experiments were performed in which multiple doses of birinapant were administered, at timepoints 0h+32h, 18h+32h and 0h+18h+32h post infection with sporozoites to test whether increasing doses and changing the timing

improved its efficacy. It was found that increasing doses of birinapant resulted in suppression of parasite load at 44h post infection, but again complete parasite clearance was not observed (Figure 11C). The most significant reductions were seen with 2 or 3 doses at the times 18h+32h and 0h+18h+32h. This may be due to the timing of TNF release following sporozoite challenge. Together, this data suggests that time of administration is not strictly important, provided that the time of administration is within a window that allows the antagonization of cIAP1 and cIAP2 levels to occur during parasite growth.

Whilst birinapant shows strong efficacy in reducing parasite liver load, some remained. To test whether these could initiate a patent infection and develop normally as asexual stages, mice were injected with 1000 sporozoites, treated with birinapant or a DMSO vehicle and blood stage parasitemia was monitored over time. The lower inoculum of parasites was chosen to more closely resemble the numbers occurring during natural malaria transmission, where a mosquito deposits only tens-to-hundreds of sporozoites. The time to first detectable patency was measured by Giemsa smears of tail vein blood and the birinapant treated group exhibited a 1-day delay ($p=0.05$, Figure 11D). Whilst not significantly different, there is a promising trend towards showing birinapant's effect of delaying patent infection. It has been reported previously that a single day delay to patency reflects an approximately 10-fold reduction in parasite liver load in *P. berghei*. This delay is consistent with the reduction in parasite load observed by qPCR.

To assess the fitness of breakthrough liver stage parasites in the blood, parasitemia measurements were made each day following infection. These results further illustrate the delay to patency and show that the birinapant treated mice took longer to develop blood stage parasitemia levels than DMSO-control mice. Indeed, the DMSO-treated animals required euthanasia when the parasitemia reached 20% or if the animals showed malaria symptoms. The birinapant-treated animals were not required to be culled due high parasitemia or severe malaria symptoms as early as DMSO-control treated mice (Figure 11E). Together these results show that while mice may still develop blood stage infection and malaria following birinapant treatment, the drug is able to clear the

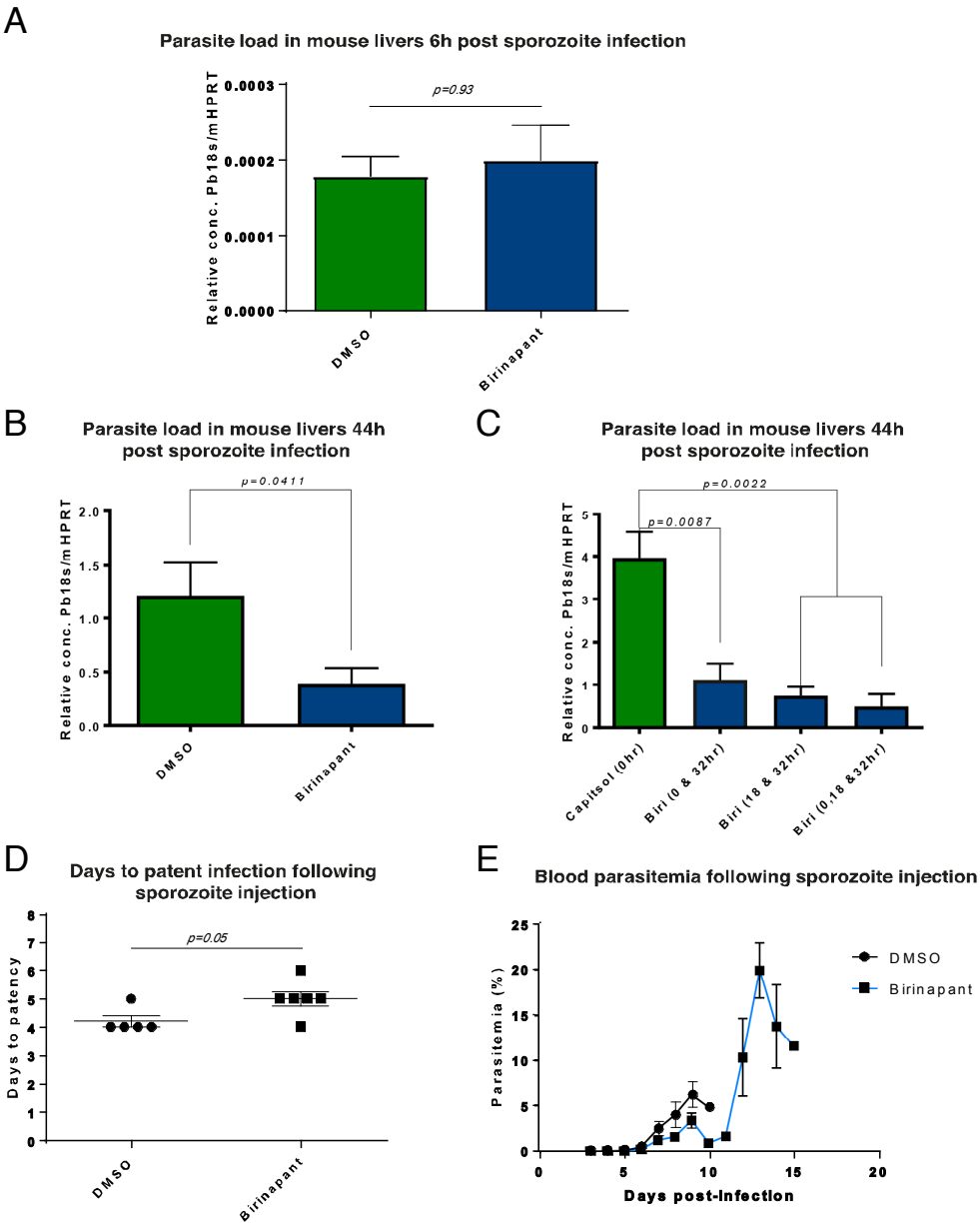


Figure 11: A. Quantification of liver parasite load 6h post infection using RT-PCR in C57Bl/6 mice treated with either DMSO (vehicle) or birinapant (40mg/kg) prior to infection with sporozoites. n=4-6 mice per group **B.** Quantification of liver parasite load 44 hour post infection using RT-PCR in C57Bl/6 mice treated with either DMSO (vehicle) or birinapant (40mg/kg) prior to infection with sporozoites. n=4-6 mice per group. **C.** Quantification of liver parasite load 44 hour post infection using RT-PCR in C57Bl/6 mice treated with either Captisol (vehicle) or birinapant (40mg/kg) at varying doses and time points around the time of infection with sporozoites. n=4-6 mice per group. **D.** Days taken for DMSO or birinapant treated (40mg/kg, 6h prior infection) mice to develop blood stage patent infection following sporozoite injection. **E.** Parasitemia growth during blood stages in DMSO/birinapant treated mice following sporozoite injection. When mice showed visual signs of sickness or high parasitemia, they were euthanized and not represented on later points in the figure.

majority of liver stage parasites causing an apparent delay in blood stage infection and survival.

Discussion

This chapter has investigated apoptosis signaling in hepatocytes and shown the importance of the cIAPs during liver stage infection. This suggested that host-based prophylaxis may be an avenue to reduce or clear liver infections and prevent malaria. There are several important factors to consider when targeting liver stage parasites with drugs. Firstly, the liver stage of the disease is clinically silent, and only blood stage malaria exhibits symptoms. Hence, diagnosis of malaria is currently limited to blood stages, meaning that targeting the liver stage with therapeutics are only relevant and effective with prophylactic administration. Secondly, killing parasites in the liver must be completely efficacious as any residual parasites able to establish blood stage disease renders the treatment ineffective. It is important to consider these issues when evaluating whether a SMAC mimetic like birinapant or one of the many others currently in clinical trials, could have use as a therapeutic. Having a drug like a SMAC mimetic that targets the host cell to kill liver stage parasites, presumably by agonizing the extrinsic pathways of apoptosis, might be useful to kill dormant hypnozoites that cause relapsing malaria and also avoid parasite drug resistance. The differences between rodent malaria (the model used in most studies) and human malaria liver infection are critical to consider in this context. *P. falciparum and P. vivax* liver stages develop over 7-10 days, whereas rodent malaria liver stages completely mature after 3 days. The longer development times for human malaria parasites may increase the therapeutic window for drugs that target host cell signaling, allowing for potentially greater clearance than can be observed in *P. berghei* mouse models. The use of humanized mice may help answer this question in future.

Other studies have used pro-apoptotic drugs to similarly show great reductions in parasite load (Douglass *et al.*, 2015; Kaushansky, Ye*, et al.*, 2013). Multiple drugs administered to target the instrinsic apoptotic pathways administered in

combination further increase the clearance observed in liver cells. Considering this, combination therapies targeting both the intrinsic and extrinsic apoptotic pathways may further increase the competency of the drugs. This would require further experimentation, and a better understanding of which effectors the parasite exports or secretes to potentially manipulate these pathways and could reveal new parasite-specific drug targets that may be more effective in eliminating liver stage parasites.

Specifically targeting host cell mechanisms also provides the additional benefit of avoiding the development of resistance to the drug. Targeting liver stage results in exposing fewer parasites to drug pressure compared to the billions of parasites that exponentially grow in the blood stage. Furthermore, targeting host processes provides less chance for parasitic mutations and variance between parasite strains to have an impact on the efficacy of the drug. With all current frontline antimalarials targeting parasitic blood stages showing signs of resistance in South-East Asia (Ashley *et al.*, 2014; Dondorp *et al.*, 2009), the usefulness of drugs that do not select for resistance quickly will be important.

Arresting liver stage parasites are known to induce protective immunity. This has been demonstrated with irradiated and genetically attenuated parasites, which are considered to have great potential for use in future vaccines (Aly *et al.*, 2011; Annoura *et al.*, 2012; Mueller *et al.*, 2005). Birinapant or other pro-apoptotic treatments could cause clearance of liver stage infection that elicits immune responses that generate a vaccine-like effect. Further experimentation is currently underway to explore this possibility.

Chapter 6: General Discussion

This dissertation aimed to investigate the export mechanisms of liver stage *Plasmodium* infection using the rodent model system *P. berghei* and investigate the processes involved in host cell death that are important for the parasite to survive in hepatocytes. Several knowledge gaps exist in the literature with respect to these questions, more specifically, these are: whether the aspartyl protease plasmepsin V is functional in *P. berghei* and whether it is essential during liver stage infection and why, whether the PEXEL motif itself is important for plasmepsin V processing in *P. berghei* during export to the erythrocyte and whether this is also important in the liver stage, and whether host cell death signalling pathways are important for the parasite to survive in hepatocytes. Despite plasmepsin V inhibitors being developed in our laboratory, we chose to utilise genetic knock down of plasmepsin V during mosquito passage and analysis of *P. berghei* mutant parasites throughout this thesis has established a new understanding of the ways the malaria parasite interacts with the host hepatocyte during infection. These advances in understanding the fundamental importance of plasmepsin V and protein export to *P. berghei* liver stages now provides the platform for future research to identify novel exported effector proteins and study their roles, and that of the PTEX translocon, in parasite-host dynamics during liver infection. The demonstration that the extrinsic host cell death signalling pathway is important to pre-erythrocytic parasite development also opens avenues for host-based prophylaxis that targets the liver stage of the parasite life cycle to clear malaria infections and induce immunity whilst circumventing drug resistance in the parasite.

Plasmepsin V: an essential enzyme with a role in export in the liver stage

An ideal method to study the potential importance of PEXEL-mediated protein export in liver stage infection would be to examine the role of plasmepsin V, given it's role in PEXEL cleavage during export. If its function is important during liver infection then the repertoire of cargo proteins, or the "exportome", during liver infection could be important, as this would provide the basis for exported hepatocyte effector proteins to be functionally analysed. This would also indicate that plasmepsin V is a drug target in both asexual and liver stages, an important criteria for new antimalarial drugs according to the Medicines for Malaria Venture.

Generating a plasmepsin V knockdown using the Flp-*FRT* system in *P. berghei* (as described in Chapter 3), has provided the tools required to begin dissect the mechanisms of protein export that occur during liver stage infection. Parasites lacking plasmepsin V arrest and do not develop to mid or late liver stages (Figure 3D) suggesting a crucial role for plasmepsin V and PEXEL mediated export in the early phase of liver stage infection. This is the first genetic evidence showing the requirement of plasmepsin V and liver stage protein export and may indicate that the parasite needs to combat host innate immune responses to survive, such as autophagy or apoptosis. This work lays the foundation to begin characterizing this important issue.

Prior knowledge of the process of liver stage protein export has been fragmented across multiple studies. The double PEXEL-containing circumsporozoite protein (CSP) has been shown to be exported into the host hepatocyte via antibody detection (Singh *et al.*, 2007), however, the images of export in the hepatocyte of that publication were very saturated suggesting CSP is exported to a very low degree, and the presence of mutant CSP containing mutated PEXEL motifs within the hepatocyte cytosol has also been reported (Cockburn *et al.*, 2011), questioning whether CSP is exported using the canonical PEXEL pathway at all. Interesting findings regarding the PTEX translocon components in liver infection

suggest that whilst the pore-forming protein EXP-2 is important for parasite survival, recently a role for this protein in solute transport across the PVM has been reported (Garten *et al.*, 2018) and so direct evidence for a role in export would be needed to understand if it contributes to export. Furthermore, the PTEX unfoldase chaperone HSP101 may not be not expressed in the liver stage parasite (Kalanon *et al.*, 2015) when proteins are exported. An explanation for this could be that the parasite may be recruiting/hijacking host cell chaperones to facilitate the (un)folding of proteins beyond the vacuole membrane or that another parasite protein is involved in unfolding. An alternative albeit unlikely possibility is that analogous to the twin-Arginine system of prokaryotes, the pre-erythrocytic forms might translocate folded proteins into the host cell. The putative lack of HSP101 from PTEX in *P. berghei* liver stages and inability to export folded tags like GFP and mCherry (see below) should be confirmed with *P. falciparum*, but provides one of potentially several differences in the export mechanism between blood and liver stages, suggesting there could be further undiscovered processes that the liver parasite might require.

Other identified PEXEL containing liver stage proteins include IBIS1 (Ingmundson *et al.*, 2012; Petersen *et al.*, 2015) and LISP2 (Orito *et al.*, 2013). IBIS1-mCherry is not exported to hepatocytes and LISP2 contains a possible PEXEL motif in an atypical region, further obfuscating whether the PEXEL motif directs export in hepatocytes at all. The discovery of the importance of plasmepsin V with the flp-*FRT* knockdown sheds light on this issue of protein export, conclusively showing that PMV is essential and by using LISP2 as a reporter, that the PEXEL motif is functional during liver infection. Initial evidence of PBANKA_0700700 being exported in the host hepatocyte (Figure 8) further supports this theory. With the possibility of an array of exported *Plasmodium* proteins to be discovered and characterised within an infected hepatocyte, further research in the area could assist to identify new drug targets and vaccine candidates to facilitate malaria eradication.

Folded reporter proteins interfere with export in to the hepatocyte

One of the challenges in identifying cases of protein export within *Plasmodium* infected hepatocytes has been visualising the proteins within the host cell. Immunofluorescent detection of liver stage proteins has been achieved only with highly expressed proteins such as CSP (Singh *et al.*, 2007) and LISP2 (Orito *et al.*, 2013). The use of reporter protein tags (such as GFP and mCherry) have been useful in identifying exported proteins in blood stages (Boddey *et al.*, 2010; Boddey *et al.*, 2013; Hiller *et al.*, 2004; Marti *et al.*, 2004) and also characterizing protein expression during liver stage infection (Fougère *et al.*, 2016).

Interestingly, all reported studies of protein export in liver stages using GFP or mCherry as a tag have observed secretion to the parasitophorous vacuole but no further trafficking. The Fam-a, Fam-b and PIR variant family of genes, when tagged with GFP, are all secreted to the vacuole but not found in the host hepatocyte cytoplasm and so were not exported (Fougère *et al.*, 2016). IBIS1 (the protein localising to Maurer's cleft-like structures in blood stage *P. berghei*) interestingly similarly only localises to the parasitophorous vacuole (Ingmundson *et al.*, 2012). Even in the case of LISP2, a protein which is clearly exported to the hepatocyte cytoplasm, tagging it with mCherry results in accumulation of the protein at the parasitophorous vacuole membrane for unknown reasons (Orito *et al.*, 2013).

This dissertation has characterised the export of the PEXEL containing gene, PBANKA_0700700, by analysing its expression during liver stage infection using an mCherry or haemagglutinin (3xHA) tagged protein. Results indicated that the export of PBANKA_0700700 was also dependent upon the type of tag used for detection. HA-tagged protein could be observed within the hepatocyte nucleus in some cells, however, fusion to an mCherry tag caused the protein to localise to the parasitophorous vacuole (Figure 8).

This result signifies the extraordinary differences that are present between the parasitic export processes that occur in the blood and liver stages of infection. These differences observed could possibly be attributed to the PTEX translocon components expressed during both stages. It is known that many cargo proteins are required to be unfolded and refolded for translocation across the parasitophorous vacuole (Gehde *et al.*, 2009). The PTEX complex features proteins essential for the translocation to occur; one of these proteins is the chaperone-like heat shock protein 101 (HSP101) (Beck *et al.*, 2014; de Koning-Ward *et al.*, 2009; Elsworth *et al.*, 2014). Interestingly, HSP101 might be absent during *P. berghei* liver stage parasite development (Kalanon *et al.*, 2015). Taken together with the PEXEL-mCherry and PEXEL-HA export data seen in liver infection, this suggests that this HSP101 chaperone may be required for the proper unfolding of the larger tag GFP/mCherry-protein complexes, although expression of this protein in liver stages in notably toxic (Kai Matuchewski, personal communication).

This significant finding suggests that future experiments intending to characterise exported proteins will require the use of smaller tags to accurately detect protein export and correct localisation. Studies analysing protein export in other Apicomplexans, such as *Toxoplasma,* may also benefit from using smaller tags as these export processes are also not well understood and may feature more similarities to *Plasmodium* liver infection than that of the blood stages, due to a larger nucleated host cell and longer developmental times. Indeed, fusion of GFP to GRA16 blocks its translocation to the host cell by Toxoplasma.

Why do malaria parasites need to export proteins during the liver stage?

In erythrocytic infection, the export of proteins to the host cell is essential due to the rigorous remodelling of the blood cell that allows the infected cell to sequester/cytoadhere to blood vessels and other tissues, evading the immune

system and causing the pathogenesis of malaria. Whilst the case for protein export occurring during liver infection is definitive, the reasons for the export mechanisms are less well understood. Unlike blood stages, the hepatocyte does not need to travel throughout the body and evade the immune system via sequestration. The disease symptoms are also completely absent. The question then remains, what is the liver stage parasite using protein export for?

Almost all cells have innate defence mechanisms that detect and eliminate invading pathogens, including hepatocytes. It is apparent from the literature that pre-erythrocytic stages protect the host cell from apoptosis and also autophagy, both of which can kill intracellular pathogens. It is tempting to speculate that liver stages export proteins that inactivate these host innate defences in order to grow inside the host cell, although direct proof of this remains to be obtained. During chapter 4, it was noted that the exported protein PBANKA_0700700 contained a nuclear localisation signal (NLS) that could be directing its export into the hepatocyte nucleus. Similar NLS sequences have been observed in exported Toxoplasma proteins such as GRA16 (Bougdour *et al.*, 2013; Coffey *et al.*, 2015; Hammoudi *et al.*, 2015), which are also directed to the host cell nucleus. It is possible that the parasite may be hijacking and manipulating host cell machinery, perhaps through the use of transcription factors and gene modulation, to enhance its growth within the liver cell.

The liver stage parasite's ability to inhibit host apoptotic mechanisms was explored using the murine genetic knockouts of the cellular inhibitors of apoptosis proteins (cIAP) and the SMAC mimetic birinapant during chapter 5. It was shown that parasite load could be controlled by priming hepatocytes for apoptosis (via cIAP1/2 knockouts). It is entirely plausible that the mechanisms the parasite uses to inhibit apoptosis may utilise the PEXEL export processes described above. As this thesis has already shown the importance of plasmepsin V and liver stage protein export, further research into identifying the function of exported proteins and establishing links between protein export and parasite survival may reveal new avenues for targeted therapeutics and attenuated parasitic vaccines.

Conclusion

The research presented in this thesis has revealed with substantial evidence the importance of plasmepsin V and protein export during *Plasmodium* liver stage development. The essentiality of plasmepsin V has been established through the generation of a conditional flp-FRT knockdown system. Further characterisation of protein export was seen with analysis of LISP2 using the knockdown line and the detection of the exported PEXEL protein PBANKA_0700700 using several different protein tags. These parasite lines developed for this dissertation are crucial tools that will prove invaluable for future research to illustrate further mechanisms behind liver stage protein export and why it occurs. It is hoped that such research will lead to the development of new antimalarials and vaccines that will aid in endeavours to eradicate malaria worldwide.

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