PTEX characterisation and its role in *Plasmodium* virulence and survival

By

Kathryn Matthews  
BSc (Hons)

Submitted in fulfillment of the requirements for the degree of  
Doctor of Philosophy (Medicine)

Deakin University  
March 2015
I am the author of the thesis entitled

PTEX characterisation and its role in *Plasmodium* virulence and survival

submitted for the degree of

Doctor of Philosophy (Medicine)

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: Kathryn Matthews

Signed: .................................................................

Date: ..............................................................................

9 June 2015
I certify the following about the thesis entitled (10 word maximum)

**PTEX characterisation and its role in *Plasmodium* virulence and survival**

submitted for the degree of

**Doctor of Philosophy (Medicine)**

a. I am the creator of all or part of the whole work(s) (including content and layout) and that where reference is made to the work of others, due acknowledgment is given.

b. The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

c. That if the work(s) have been commissioned, sponsored or supported by any organisation, I have fulfilled all of the obligations required by such contract or agreement.

I also certify that any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

'I certify that I am the student named below and that the information provided in the form is correct'

**Full Name:** Kathryn Matthews

**Signed:** .................................................................

**Date:** 11 March 2015

Signature Redacted by Library
Acknowledgement

The work undertaken within this project was only feasible with the on-going guidance, and all-round support from my supervisor, Associate Professor Tania de Koning-Ward. Her help with matters in the lab, right through to the end of this thesis has been extraordinary. Her persistence and encouragement has always been strong, and she is a brilliant role model. For all this, I am truly grateful.

My associate supervisor, Dr Ming Kalanon, has been a guiding light throughout this project. His world of knowledge exceeds most, and his scope for creativity matches. I have been extremely fortunate to be surrounded with a positive and vibrant mentor, to show me where and how to improve as a research scientist.

Whilst not formally linked to this project, the members of the TDK-W lab have been supportive on many levels, and without your contributions, the days would have been harder to get through.

I would also like to acknowledge the contribution that has been made as a recipient of an Australian Postgraduate Research Award, without which, none of this work would have been possible.
List of publications

Published scientific publications resulting from studies undertaken and described in this thesis are the following:


Abstract

During the blood stages of malaria, *Plasmodium* parasites remodel their vertebrate host cells by translocating hundreds of parasite-encoded proteins across an encasing double-membrane barrier that separates the parasite from the host cell cytosol via a putative export machinery termed PTEX\(^1\). Exported proteins have a variety of roles that have been linked to parasite growth and virulence. These include proteins containing the PEXEL motif (RxLxE/Q/D\(^{1,2}\)) as well as PEXEL-negative exported proteins (PNEPs\(^6\)). Whilst PTEX has been linked to the export of PEXEL-positive exported proteins, there has yet been no direct evidence that PTEX is responsible for the translocation of exported proteins. Previously, PTEX has been shown to comprise of three core components, those being PTEX150, HSP101 and EXP2. Two other proteins, namely TRX2 and PTEX88 had been tentatively assigned to PTEX but required validation that they are bona fide PTEX components.

As a result of this study, it was confirmed that PTEX additionally comprises of TRX2 and PTEX88, and which the components of PTEX are essential or non-essential to parasite survival was also revealed. As a non-essential PTEX component, TRX2 could be genetically deleted, although parasites transgenic parasites lacking TRX2 were significantly affected in their growth. As viable parasites harboring a TRX2 gene deletion could be recovered, the parasites ability to export proteins could be dissected. While the export of individual proteins or reported proteins did not appear to be compromised, the cumulative effect of protein export was, with TRX2 deficient parasites showing a significant reduction in export of proteins to the infected erythrocyte surface. In contrast, knockdown of the essential protein
HSP101, had significant consequences on protein export with both classes of exported proteins affected. This indicates targeting of PTEX with anti-malaria drugs should be against essential PTEX components.
Table of Contents

Title Page.................................................................i
Declaration............................................................ii
Acknowledgement....................................................iii
List of publications....................................................iv
Abstract.......................................................................v
Table of Contents......................................................vi
List of Tables............................................................ix
List of Figures............................................................x
List of Abbreviations...................................................xii

Chapter 1.............................................................................1

Literature Review ............................................................1
1.1 Introduction – The burden of malaria .......................2
1.2 The life cycle of Plasmodium....................................2
  1.2.1 Transmission of Plasmodium to the human host involves two hosts........2
  1.2.2 Parasite invasion of the host erythrocyte............................3
  1.2.3 Parasite development within the erythrocyte ......................4
    1.2.3.1 Parasite growth during the asexual stages....................4
    1.2.3.2 Sexual stage development is essential for Plasmodium
      transmission to the mosquito............................................5
  1.2.4 Pathogenesis of malaria .......................................7
1.3 Combating malaria – past, present and future treatments ...........8
  1.3.1 Existing anti-malaria prophylaxis and treatment ..............9
  1.3.2 The future of malaria treatment ................................11
1.4 Remodeling of the host erythrocyte..............................11
  1.4.1 Morphological and structural changes to the host erythrocyte ..........12
  1.4.2 Protein export....................................................15
    1.4.2.1 Protein export and the role of the PEXEL/HT motif in protein
      export........................................................................16
    1.4.2.2 PEXEL containing exported proteins............................17
    1.4.2.3 PEXEL Negative Exported Proteins (PNEPs)..................20
1.5 Protein translocation across the PVM...........................21
1.5.1 The *Plasmodium* Translocon of Exported Proteins (PTEX) ........................................ 23
1.6 Project aims and hypothesis .................................................................................... 28

Chapter 2 ...................................................................................................................... 30

Materials and Methods ................................................................................................ 30
1.7 Materials .................................................................................................................. 31
1.8 Methods .................................................................................................................... 32
  1.8.1 Ethics statement ................................................................................................. 32
  1.8.2 DNA and protein sequence analysis .................................................................. 32
  1.8.3 Oligonucleotide Primers .................................................................................. 32
  1.8.4 DNA applications .............................................................................................. 32
    1.8.4.1 DNA preparation ....................................................................................... 32
    1.8.4.2 Polymerase Chain Reaction (PCR) amplification ...................................... 33
    1.8.4.3 DNA digestion ........................................................................................... 33
    1.8.4.4 DNA ligation .............................................................................................. 33
    1.8.4.5 Bacterial transformation ............................................................................. 34
    1.8.4.6 DNA sequencing ....................................................................................... 34
    1.8.4.7 Agarose gel electrophoresis ....................................................................... 34
    1.8.4.8 Southern blot hybridisation ....................................................................... 35
      1.8.4.8.1 Preparation of membrane ................................................................... 35
      1.8.4.8.2 Digoxigenin labeled DNA probes ...................................................... 35
      1.8.4.8.3 Hybridisation ..................................................................................... 36
      1.8.4.8.4 Detection ............................................................................................ 36
      1.8.4.8.5 Stripping probes from membrane ...................................................... 37
  1.8.5 Plasmid constructs ............................................................................................... 37
    1.8.5.1 Epitope tagging ......................................................................................... 37
    1.8.5.2 PTEX gene deletion ................................................................................... 38
  1.8.6 Protein applications ............................................................................................ 39
    1.8.6.1 Protein expression ..................................................................................... 39
    1.8.6.2 Protein purification .................................................................................... 39
    1.8.6.3 Protein electrophoresis .............................................................................. 40
    1.8.6.4 Western blot analysis ................................................................................. 40
    1.8.6.5 Protein immunoprecipitations ................................................................... 41
    1.8.6.6 Gel excision and Nano-LC-MS/MS ........................................................... 42
1.8.6.7 Recombinant protein purification from maltose binding protein ................................................................. 43

1.8.7 Plasmodium applications ................................................................................................................................. 44
1.8.7.1 Infection of mice with *P. berghei* .................................................................................................................... 44
1.8.7.2 Preparation of DNA for *P. berghei* transfection .......................................................................................... 44
1.8.7.3 Preparation of schizonts for *P. berghei* transfection .................................................................................... 45
1.8.7.4 Transfection and drug selection ...................................................................................................................... 45
1.8.7.5 Isolation of parasite DNA from whole blood .................................................................................................. 46
1.8.7.6 Cloning of transgenic parasites ..................................................................................................................... 47
1.8.7.7 Parasite growth curves ..................................................................................................................................... 47
1.8.7.8 Parasite virulence analysis ............................................................................................................................. 47
1.8.7.9 *In vivo* / *in vitro* growth assay .................................................................................................................... 48
1.8.7.10 Generation of semi-immune sera .................................................................................................................. 48
1.8.8 Immunofluorescence analysis of *P. berghei* ....................................................................................................... 48
1.8.9 Flow Cytometry .................................................................................................................................................. 49
1.8.10 Statistical Analysis ........................................................................................................................................... 50

Chapter 3 ............................................................................................................................................................................ 53

The conservation and characterisation of the Plasmodium Translocon of Exported Proteins ............................................................... 53

1.9 Introduction ............................................................................................................................................................ 54
1.10 Results ..................................................................................................................................................................... 55
1.10.1 PTEX components are expressed in *P. berghei* ............................................................................................... 55
1.10.2 Generation of reagents to *P. berghei* PTEX components .................................................................................. 56
1.10.2.1 Attempts to generate antibody reagents against PTEX150 and TRX2 .......................................................... 57
1.10.2.2 Peptide antibody development against TRX2 ............................................................................................... 60
1.10.2.3 Epitope tagging of PTEX components ........................................................................................................... 61
1.10.3 *P. berghei* PTEX orthologues localise to the PVM ......................................................................................... 63
1.10.4 *P. berghei* TRX2 and PTEX88 interact with other PTEX components ........................................................................... 65
1.11 Discussion ............................................................................................................................................................... 68

Chapter 4 ............................................................................................................................................................................ 85
Dissecting the role of the PTEX components and assessing parasite growth and virulence............................................................................................................ 85

1.12 Introduction .................................................................................................... 86
1.13 Results ........................................................................................................... 87
   1.13.1 Generation of PTEX knockout constructs ............................................... 87
   1.13.2 Analysis of P. berghei transfectants ........................................................ 88
   1.13.3 The P. berghei TRX2 gene can be genetically deleted .......................... 90
   1.13.4 Creation of clonal P. berghei TRX2 gene knock out parasites ............... 90
   1.13.5 Attempts to complement the P. berghei TRX2 knockout parasites ...... 91
   1.13.6 The loss of TRX2 affects P. berghei growth in vivo ................................. 93
   1.13.7 Assessment of PbΔTRX2 growth in vitro ................................................ 95
   1.13.8 Loss of TRX2 leads to alterations in parasite virulence .......................... 96
1.14 Discussion .................................................................................................... 98

Chapter 5............................................................................................................... 112

Functional evidence that PTEX is involved in parasite protein export .......... 112

1.15 Introduction .................................................................................................. 113
1.16 Results ......................................................................................................... 113
   1.16.1 The deletion of P. berghei TRX2 deletion does not affect the export of the endogenous PEXEL proteins PBANKA_122900 & PBANKA_114540 ............................................................................... 113
   1.16.2 P. berghei TRX2 knockout parasite lines expressing an episomally-expressed exported reporter protein ......................................................... 114
   1.16.3 Generation of a P. berghei TRX2 knockout parasite line containing an integrated exported GFP reporter gene ............................................. 116
   1.16.4 Live-cell fluorescent imaging of wild type or TRX2 knockout parasites expressing an exported GFP reporter ................................................. 118
   1.16.5 Generation of parasites harboring an exported Nanoluc reporter construct ..................................................................................................... 120
   1.16.6 Measurement of global protein export in P. berghei using semi-immune sera .............................................................................................. 122
   1.16.7 Structural and morphological changes to P. berghei infected erythrocytes ................................................................................................. 125
1.17 Discussion .................................................................................................... 127
List of Tables

Chapter 1
Table 1.1: Comparison of the *P. falciparum* & *P. berghei* exportome

Chapter 2
Table 2.1: Oligonucleotides used in this study

Chapter 3
Table 3.1: Comparisons between the *P. falciparum* and the orthologous *P. berghei* PTEX protein
Table 3.2: Reactivity of *P. falciparum* antibodies
List of Figures

Chapter 1

Figure 1.1: The life cycle of the *Plasmodium* parasite.

Figure 1.2: Schematic model of PTEX.

Chapter 3

Figure 1.1: Schematic of the *P. falciparum* PTEX components and the orthologous proteins in *P. berghei*.

Figure 3.2: Anti-PfHSP101 and anti-PfEXP2 cross-react with *P. berghei*.

Figure 3.3: Expression of recombinant PTEX150 and TRX2 protein.

Figure 3.4: Cleavage of recombinant PTEX150 and TRX2 protein.

Figure 3.5: Sequence alignment between *P. falciparum* and *P. berghei* TRX2.

Figure 3.6: Generation of *P. berghei* transgenic parasites expressing epitope tags.

Figure 3.7: Expression of *P. berghei* transgenic parasites with epitope tags.

Figure 3.8: *P. berghei* PTEX components colocalise and have PV/PVM localization.

Figure 3.9: The five putative PTEX components form a complex in *P. berghei*, including PTEX88 and TRX2.

Figure 3.10: PbTRX2-HA interacts with unique protein species.

Figure 3.11: PbTRX2-HA associates with PTEX & non-PTEX proteins.

Chapter 4

Figure 4.1: Construction of pB3-PTEX knockout constructs.
Figure 4.2: HSP101, PTEX150, EXP2 and PTEX88 are not amenable to gene deletion

Figure 4.3: Genetic disruption of TRX2 *P. berghei*.

Figure 4.4: Confirmation of the *P. berghei* TRX2 gene knockout

Figure 4.5: Construction of a *P. berghei* TRX2 complement plasmid.

Figure 4.6: Loss of TRX2 in *P. berghei* leads to reduced parasite loads *in vivo*.

Figure 4.7: Loss of TRX2 in *P. berghei* results in a longer parasite cycle *in vivo*.

Figure 4.8: Loss of TRX2 leads to altered growth phenotypes *in vitro*.

Figure 4.9: Loss of TRX2 in *P. berghei* leads to altered virulence and growth phenotypes in C57/Bl6 mice.

**Chapter 5**

Figure 5.1: *P. berghei* TRX2 deletion does not affect the ability to export two identified endogenously expressed exported PEXEL proteins.

Figure 5.2: Generation of a *P. berghei* TRX2 knockout encompassing a GFP reporter.

Figure 5.3: Time-course of GFP expression and export in transgenic parasites.

Figure 5.4: Schematic of the constructs designed for episomal expression of Nanoluc in *P. berghei*.

Figure 5.5: Generation of semi-immune sera against *P. berghei* infection.

Figure 5.6: Disruption of *P. berghei* TRX2 leads to reduced protein export on the erythrocyte surface.

Figure 5.7: Disruption of *P. berghei* TRX2 leads to reduced protein export in synchronous infections.

Figure 5.8: Surface modification of infected erythrocytes
List of Abbreviations

Common scientific abbreviations

Å  angstrom
ATP  adenosine triphosphate
BSA  bovine serum albumin
DNA  deoxyribonucleic acid
ER  endoplasmic reticulum
GFP  green fluorescent protein
HRP  horseradish peroxidase
Hr/hrs  hour/hours
Hsp  heat shock protein
ICAM-1  intercellular adhesion molecule-1
IFA  immunofluorescence analysis
kb  kilobase(s)
kDa  kilodalton(s)
l  litre(s)
M  Molar
μg  microgram(s)
mg  milligram(s)
mL  millilitre(s)
mM  millimolar
min  minute(s)
MW  molecular weight
nm  nanomole(s)
% (v/v)  percent volume per volume
% (w/v)  percent weight per volume
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
rpm  revolutions per minute
RT  room temperature
s  second(s)
SDS  sodium dodecyl sulphate
SEM  standard error of the mean
TBE  Tris-borate-EDTA buffer
TIC  translocon at the inner membrane of chloroplasts
TOC  translocon at the outer membrane of chloroplasts
TOM  translocon at the outer membrane of mitochondria
TIM  translocon at the inner membrane of mitochondria
UTR  untranslated region

Plasmodium parasite specific abbreviations

ACT  artemisinin-based combination therapies
EDV’s  electron-dense vesicles
EMAP  erythrocyte membrane associated protein 1
GBP  glycophorin-binding protein-130
GPI  glycosylphosphatidylinositol
HSP  heat shock protein
HT  Host-Targeting Signal
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAHRP</td>
<td>knob-associated histidine rich protein</td>
</tr>
<tr>
<td>MAHRP-1</td>
<td>membrane-associated histidine-rich protein 1</td>
</tr>
<tr>
<td>MAHRP2</td>
<td>membrane-associated histidine-rich protein 2</td>
</tr>
<tr>
<td>mDHFR</td>
<td>mouse dihydrofolate reductase</td>
</tr>
<tr>
<td>NPPs</td>
<td>new permeation pathways</td>
</tr>
<tr>
<td>PEXEL</td>
<td><em>Plasmodium</em> EXport EElement</td>
</tr>
<tr>
<td>Pf</td>
<td><em>Plasmodium falciparum</em></td>
</tr>
<tr>
<td>Pb</td>
<td><em>Plasmodium berghei</em></td>
</tr>
<tr>
<td>PfEMP1</td>
<td><em>P. falciparum</em> erythrocyte membrane protein</td>
</tr>
<tr>
<td>PIR</td>
<td><em>Plasmodium</em> interspersed repeats</td>
</tr>
<tr>
<td>PNEPs</td>
<td>PEXEL negative exported proteins</td>
</tr>
<tr>
<td>PTEX</td>
<td><em>Plasmodium</em> Translocon of EXported proteins</td>
</tr>
<tr>
<td>PV</td>
<td>parasitophorous vacuole</td>
</tr>
<tr>
<td>PVM</td>
<td>parasitophorous vacuole membrane</td>
</tr>
<tr>
<td>RESA</td>
<td>ring-expressed surface antigen</td>
</tr>
<tr>
<td>REX1</td>
<td>ring exported protein 1</td>
</tr>
<tr>
<td>REX2</td>
<td>ring exported protein 2</td>
</tr>
<tr>
<td>Rif</td>
<td>Repetitive interdispersed family</td>
</tr>
<tr>
<td>SBP-1</td>
<td>skeleton-binding protein 1</td>
</tr>
<tr>
<td>Stevor</td>
<td>subtelomeric variable open reading frames</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review
1.1 Introduction – The burden of malaria

Malaria is a global disease caused by infection with Apicomplexan protozoan parasites of the genus Plasmodium. Multiple species of Plasmodium exist, five of which can infect humans: P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi. The latter circulates in primates but has recently been observed to also infect the human host\textsuperscript{2,3}. Whilst P. vivax is widely distributed and causes a greater number of infections\textsuperscript{4}, P. falciparum is the most virulent of the species, and is responsible for the majority of deaths caused by malaria\textsuperscript{5}; accordingly, it is the focus of this review. In 2012, an estimated 207 million malaria infections occurred, with 627,000 (±150,000) deaths attributed to severe malaria, 90\% of which occurred in sub-Saharan Africa. Most malaria infections occur in underdeveloped regions, with ~3.4 billion people at risk of infection\textsuperscript{6}. In addition to mortality and morbidity, malaria also causes an enormous financial impact on the economy, as a result of treatment costs and lost productivity.

1.2 The life cycle of Plasmodium

1.2.1 Transmission of Plasmodium to the human host involves two hosts

The spread of the malaria parasite is attributed to the female Anopheles mosquito. Infection of a human host occurs when an infected mosquito takes a blood meal and subsequently injects extracellular sporozoites present in her salivary glands into the host (Figure 1.1i)\textsuperscript{7,8}, where they migrate via the
bloodstream, passing through host cell barriers to the liver (Figure 1.1ii). There they bind to liver-specific heparan sulfate proteoglycans on the endothelial cell surface\textsuperscript{9,10} and glide along the endothelium. The parasites then actively cross the capillary wall through Küpffer cells, and transmigrate through several hepatocytes before finally invading a hepatocyte and becoming encased within a parasitophorous vacuole (PV)\textsuperscript{11}. At this stage of the malaria infection, no clinical symptoms are experienced. After parasite growth and mitotic division within the infected hepatocyte, a large schizont containing thousands of liver merozoites matures and ruptures, releasing infective merozoites into the bloodstream in vesicles called merosomes\textsuperscript{12}. The released merozoites then invade red blood cells.

**1.2.2 Parasite invasion of the host erythrocyte**

The invasion of merozoites into erythrocytes (Figure 1.1iii) relies on an initial low-affinity association between glycosylphosphatidylinositol (GPI)-anchored proteins on the surface of the extracellular merozoite and the erythrocyte surface. *Plasmodium* parasites actively penetrate their host cell, which involves a highly coordinated cascade of events that lead to the sequential secretion of proteins from the apical organelles: micronemes, rhoptries and dense granules\textsuperscript{13,14}. This includes specific interactions between host cell receptors and parasite ligands that are released from the micronemes and rhoptry, and the establishment of a tight junction at the parasite-host cell plasma membrane interface\textsuperscript{15}. Invagination is initiated and accomplished with the aid of the merozoite derived actin-myosin motor\textsuperscript{13}, with the shedding of merozoite surface proteins as the parasite moves into
the erythrocyte. Upon invasion, the parasite becomes encased inside a PV which is bounded by the parasitophorous vacuole membrane (PVM). Once invasion is complete and the PVM is sealed, the dense granules are released into the PV space. At this stage, the parasite is equipped with its core organelles, including a nucleus, endoplasmic reticulum, a rudimentary Golgi, a single mitochondrion and an apicoplast.

1.2.3 Parasite development within the erythrocyte

1.2.3.1 Parasite growth during the asexual stages

*P. falciparum*-infected erythrocytes are visible by microscopy in Giemsa-stained blood smears, where they exhibit an indicative ring-shaped appearance shortly after erythrocyte invasion. The parasite then undergoes a period of growth during the trophozoite phase of its development while actively remodeling its host erythrocyte and consuming the erythrocytes cytoplasm. For this, the food vacuole is a major parasite digestive organelle which degrades host-derived hemoglobin to obtain amino acids. A by-product of hemoglobin degradation, toxic ferrirriprotoporphyrin IX is produced which is then polymerized to crystalline hemozoin (malaria pigment), appearing as a distinct feature of the food vacuole of trophozoite and schizont parasites. Whilst hemoglobin degradation by a multitude of peptidases is sufficient to provide some amino acids, exogenous amino acids for parasite growth are also required.

Further parasite adaptations to surviving the intra-erythrocytic stage include new induced structures and morphological alterations to the
erythrocyte cytosol and the surface, which alters the rigidity of the host cell as well as its permeability, that enables the parasite to take up the necessary nutrients to support parasite growth. As discussed in section 1.4, these modifications are also important to parasite virulence and immune evasion.

During the schizont stage, the parasite undergoes numerous rounds of binary division, until finally each schizont contains between 8 and 20 daughter merozoites. Simultaneously, degradation of the erythrocyte membrane and subsequent osmotic swelling causes elastic instability of the membrane and complete membrane degeneration, leading to parasite rupture and rapid ejection of merozoites into the bloodstream, which can then infect new erythrocytes. The amplification of the erythrocyte stages and parasite rupture coincides with the symptomatic stages of infection.

1.2.3.2 **Sexual stage development is essential for *Plasmodium* transmission to the mosquito**

The asexual replication of the malaria parasite is responsible for the continual proliferation of the parasite, yet in order for disease transmission to occur between hosts, intraerythrocytic differentiation and development of highly specialized male and female gametocytes must take place (Figure 1.1iv). Gametocytogenesis begins at approximately 7–15 days after blood stage parasites appear, and is influenced by different kinds of stresses, including parasite density, host anemia, and host immune response or drug treatment. Development into gametocytes is defined early at the invading merozoite stage. Gametocyte maturation proceeds through five defined morphological stages; the first four adhere to host tissues and are
sequestered in the hematopoietic system\textsuperscript{36}. Mature, stage five gametocytes are released into the peripheral circulation\textsuperscript{34,37-39} and are taken up by a mosquito during her blood meal\textsuperscript{40,41}. Once ingested, the male and female gametocytes undergo gametogenesis within the lumen of the mosquito midgut, after which, they undergo syngamy to fuse into a zygote that by meiosis, develops into the motile ookinete. The ookinete transverses the peritrophic membrane, to ultimately reside in the extracellular space of the basal lamina. Asexual sporogony occurs and after two weeks incubation, thousands of sporozoites are produced within this oocyst. Upon maturation, sporozoites are released, after which they attach and invade the salivary gland epithelium to form a transient vacuole. Here the sporozoites mix with mosquito saliva, where few enter the salivary duct and are injected into the next vertebrate host during the next feed to perpetuate the cycle of infection\textsuperscript{42}.

\textbf{Figure 1.1: Schematic of the life cycle of} \textit{Plasmodium} \textit{parasite.}

The malaria parasite life cycle involves two hosts. Transmission of the vertebrate host is attributed to the female \textit{Anopheles} mosquito vector. i-iv refers to the phases of parasite development in the vertebrate host (see in text). ET: Early trophozoite (ring-like appearance), LT: Late trophozoite, S: Schizont.
1.2.4 Pathogenesis of malaria

As outlined in section 1.4, during the erythrocytic stages of infection, major structural and morphological changes are made to the infected erythrocyte, leading to altered physical properties and impaired cell function\textsuperscript{43}. Infected erythrocytes become rigid with a rough surface, attributed to the protrusion of small electron dense knob-like structures\textsuperscript{44} that comprise of the knob-associated histidine rich protein (KAHRP)\textsuperscript{45,46}. These protrusions act as platforms for anchoring the major virulence determinant and cytoadherence factor, \textit{P. falciparum} erythrocyte membrane protein (PfEMP1)\textsuperscript{47,48}. This mediates an adhesive phenotype on the infected erythrocyte, which enables infected cells to bind to the microvascular endothelium by interaction with host cell receptors such as CD36\textsuperscript{49-51} in most organs, intercellular adhesion molecule-1 (ICAM-1) which is predominantly present in brain tissue, and chondroitin sulphate-A in the placenta\textsuperscript{52,53}. The adhesive phenotype of the infected erythrocyte plays an important role in immune avoidance. By sequestering in the microvasculature, the infected cells avoid passage through the spleen where splenic macrophages would normally recognise and destroy altered or damaged erythrocytes\textsuperscript{54}. Infected erythrocytes can also adhere to uninfected cells in a process called rosetting\textsuperscript{55,56}, via the CD36 and platelet receptor (yet to be identified) with cell clumping likely to cause microvascular blockages and sequestration of infected erythrocytes leading to reduced microvascular blood flow and hypoxia\textsuperscript{57}. The release of \textit{Plasmodium} toxin (GPI-toxin) also stimulates macrophages to release Tumor Necrosis Factor-\alpha and other cytokines such as Interleukin-1 that contribute to the inflammatory process\textsuperscript{58}. Excess nitric oxide production is also induced, which diffuses through the blood-brain barrier leading to changes in synaptic
function and subsequently reduced consciousness\textsuperscript{59-61}. As a consequence of these mechanical and inflammatory events in the brain, seizures, paralysis and coma are common symptoms of severe cerebral malaria (CM)\textsuperscript{62}, which can be fatal. While CM is a more common presentation of severe malaria where transmission intensity is low, severe anemia predominates where transmission intensity is high\textsuperscript{5}. In addition to the destruction of infected and uninfected erythrocytes, anemia is also caused by inadequate host responses such as decreased erythrocyte production and/or suppression of the erythropoietic response resulting in ineffective erythropoiesis\textsuperscript{63}. Furthermore, metabolic acidosis\textsuperscript{49} and acute respiratory distress syndrome are other complications arising from severe malaria symptoms\textsuperscript{52}.

1.3 Combating malaria – past, present and future treatments

Individuals in \textit{Plasmodium falciparum} endemic areas slowly develop natural semi-immunity to malaria after repeated exposure to \textit{Plasmodium} infections\textsuperscript{64,65}, yet complete protective immunity is never achieved. Adaptive protection against severe malaria results in a lower risk of clinical disease, as indicated by both the absence of an accompanying fever, and lower densities of parasitemia\textsuperscript{66}. Knowledge of the acquisition and nature of protective immune responses against \textit{P. falciparum} is presently limited, particularly among young children\textsuperscript{64}, whom are most susceptible to severe malaria and subsequently endure the most fatalities\textsuperscript{67,68}. Consequently, it is paramount that individuals are protected from infection through other means, or have access to cheap and effective treatments.
1.3.1 Existing anti-malaria prophylaxis and treatment

Basic prevention of transmission with the use of insecticide-treated mosquito nets and control of the mosquito vector by insecticide spraying has proved to be an effective method in reducing the number of new infections\textsuperscript{69}. However, whilst the use of insecticide-treated bed nets and indoor residual spraying of insecticide\textsuperscript{70} has contributed to a reduction in infection rates, challenges arise with variances in compliancy\textsuperscript{49,71} and increasing resistance of the mosquito vectors to pyrethroids and to other insecticides, which overall, jeopardizes the global malaria control efforts\textsuperscript{6}. As a consequence, further prophylaxis methods for prevention have been explored, with a vaccine the most desirable option, as it would provide a greater coverage and long-term protection against malaria.

Attenuated, subunit, recombinant and DNA vaccines have all been investigated as strategies to prevent malaria infection or severity of disease. The multiple life cycle stages of the malaria parasite can provide targets for vaccine development, such as the pre-erythrocytic stages, where vaccines are directed against the invasive sporozoite. Alternatively, vaccines can be designed against the asexual blood stage where they may prevent erythrocyte invasion, destroy intra-erythrocytic parasites or reduce the virulence of the parasite and hence reduce malaria symptoms. Finally, vaccines against the sexual stage parasite to prevent intra-mosquito development is another strategy that can block transmission of malaria between human hosts\textsuperscript{72}. However, to date, vaccine development against \textit{Plasmodium} has proven to be extremely difficult due to the parasite’s ability to switch the antigens it expresses and the diverse polymorphisms exhibited in its antigens\textsuperscript{73}. For efficient eradication of malaria, a successful vaccine
would need to be at least 50% effective against severe disease and provide long lasting immunity against infection. To date, the most successful vaccine candidate, targeting the central repeat region at the carboxy-terminal of *P. falciparum* circumsporozoite protein, and termed RTS,S/AS01, has been shown to reduce mild and severe disease after preliminary stage III clinical trials. During 18 months of follow-up, vaccination of children and young infants with RTS,S/AS01 prevented many cases of clinical and severe malaria. Children receiving three doses of their first vaccination between 5-17 months of age, had a 36% reduced incidence of severe disease, while infants first vaccinated at 6–12 weeks old had a decreased incidences of severe disease of 15%. Despite this positive outlook, this vaccine is only partially protective against disease and wanes over time, with the status of efficacy of RTS,S/AS01 at the phase 3 trial lower than that observed in a phase 2 trial involving infants. Thus, the RTS,S/AS01 vaccine alone is unlikely to reduce the global burden of malaria. Therefore, to have a significant impact, even if this vaccine were to be licensed, it would need to be used in conjunction with other prevention methods.

Once an infection is diagnosed, prompt administration of appropriate drug treatments can reduce the severity or clear a malaria infection. However, past use of anti-malarial drugs has only been partially effective in controlling malaria and the high prevalence of multidrug-resistant *P. falciparum* parasites has given rise to its success as a pathogen. Primary anti-malarials that had been effective in controlling malarial infections include chloroquine and sulphadoxine-pyrimethamine. However, resistance to both drug classes has developed in most *P. falciparum* endemic areas, and as a
consequence, the use of artemisinin-based combination therapies is now recommended. The effects of artemisinin results in rapid parasite clearance with a longer-acting partner drug\textsuperscript{83} to multiple parasite stages\textsuperscript{83-85}. However, the efficacy of artemisinins has become compromised, with artemisinin resistance recently emerging, characterised by slow parasite clearance\textsuperscript{86,87}.

1.3.2 The future of malaria treatment

As parasites continue to exhibit multi-drug resistance to the last-line of treatments available, it is very clear that new strategies to combat the \textit{Plasmodium} pathogen are needed. In order to approach the development of vaccines and new drugs rationally, we require an in-depth understanding of malaria parasite biology and how \textit{Plasmodium} parasites are able to successfully evade the host immune response to cause disease. One of the processes which is central to parasite virulence and survival is the parasite’s ability to remodel its host cell. Thus understanding the mechanisms by which the parasites achieves this may identify new molecular pathways that can be targeted by drugs or a vaccine to ultimately destroy parasites at the blood stages of growth or even to reduce the severity of the disease.

1.4 Remodeling of the host erythrocyte

Healthy erythrocytes are flexible, flattened, circular discs about 6-8μm in diameter\textsuperscript{88}. Mature erythrocytes lack cell organelles, an intracellular membrane system and a protein synthesis system. They consist of a cytoplasm within a plasma membrane, containing a high concentration of
hemoglobin, which binds and releases oxygen and carbon dioxide\(^{89}\). The plasma membrane is anchored to a network of proteins known as the spectrin membrane skeleton, which gives the erythrocyte its elasticity and flexibility to allow passage through blood vessels and thin capillaries\(^{90}\). Healthy erythrocytes circulate for three months, after which old or damaged cells are cleared by the liver's reticuloendothelial system\(^{91}\). However, upon infection with \textit{Plasmodium} parasites, the erythrocyte becomes drastically remodeled. This involves structural changes to the host cell (see section 1.4.1) as well as the export of hundreds of parasite proteins (see section 1.4.2) beyond the PV and across the PVM into the host cell. These parasite proteins may either reside within the host cell cytosol as soluble proteins, or localise within or associate with novel parasite induced membranous structures in the host cell, while others are presented on the erythrocyte surface. To traffic proteins to these destinations, the parasite needs to employ its own protein export machinery to compensate for the lack of trafficking machinery in the naïve erythrocytes.

1.4.1 Morphological and structural changes to the host erythrocyte

There are several types of modifications that occur to the host erythrocyte once they become infected by \textit{Plasmodium spp.} As part of the host cell modification process, for example, membranous structures appear in the cytoplasm of infected erythrocytes\(^{92}\). In \textit{P. falciparum}, these structures are known as Maurer’s clefts, which are thought to function as a sorting compartment for parasite proteins transiting between the parasite and erythrocyte membrane\(^{93}\). While the origin of Maurer’s clefts remains
unknown, they may exist as independent structures or represent subdomains of the tubulovesicular network that extends from the PV\textsuperscript{93}. The morphology of Maurer’s clefts is heterogeneous in size and structure, consisting of both branched membrane tubules and stacked disk-shaped cisternae\textsuperscript{94}. Membranous structures have also been observed in other \textit{Plasmodium} species such as \textit{P. berghei}\textsuperscript{95,96} but whether these perform a similar function to Maurer’s clefts is unknown.

The formation and maturation of Maurer’s clefts in \textit{P. falciparum} has been examined in trafficking studies combined with 4D microscopy/tomography using integral Maurer’s cleft proteins fused to fluorescent proteins as markers. These experiments have shown that clefts are present much earlier than initially thought, appearing 2 - 4 hrs post invasion\textsuperscript{97}, and that a set number of Maurer’s clefts are produced after invasion, with no new structures appearing over the remaining stages of parasite development\textsuperscript{97}. This indicates that Maurer’s clefts are generated before most proteins destined for export have been synthesized. However, as proteins localise to the Maurer’s clefts after their formation, suggests that the major route of export for membrane proteins is not during cleft formation but via alternative pathways\textsuperscript{97} such as vesicles\textsuperscript{98} or soluble intermediates\textsuperscript{99}. Further to this, Maurer’s clefts maintain a fixed position within the host cytoplasm due to tethers\textsuperscript{100} and thin attachments made between the erythrocyte membrane and PVM, although no continuous trafficking network to allow transport of proteins or molecules exists between the three compartments\textsuperscript{93,101,102}.

Parasite derived ‘dot-like’ structures termed J-dots\textsuperscript{103}, are also distributed within the host cell cytoplasm in the early ring stage of the \textit{P. falciparum} blood stage life cycle. J-dots are also potentially membrane vesicles, but
these do not co-localise with the Maurer’s cleft marker, Skeleton-binding protein 1 (PfSBP1), nor do they share any other of the Maurer’s clefts antigenic determinants\textsuperscript{103}. Proteins that have been shown to associate with J-dots include molecular chaperones and co-chaperones, which may facilitate the export and folding of other parasite-encoded proteins in the infected erythrocyte cytosol\textsuperscript{103,104}.

Another major structural change that has only been described in \textit{P. falciparum}-infected erythrocytes is the development of electron-dense protrusions or knobs on the membrane surface\textsuperscript{105}. These structures are formed by KAHRP and erythrocyte membrane protein-2 (PfEMP2) also known as MESA, which localises to the cytoplasmic side of the erythrocyte membrane. The knobs are responsible for anchoring PfEMP1, and thus contribute to sequestration of infected erythrocytes and parasite virulence\textsuperscript{106}, as outlined in section 1.2.4. Plasmodium species other than \textit{P. falciparum}, such as rodent malaria \textit{P. berghei} also cytoadhere to the endothelial lining in the microvasculature\textsuperscript{107,108}, but none of these species harbour PfEMP1\textsuperscript{109} or KAHRP orthologues. Thus what parasite molecules mediates the binding to host ligands, such as CD36\textsuperscript{107} in these species remains unknown.

Other parasite induced structures observed in \textit{P. falciparum}-infected erythrocytes include large electron-dense vesicles (EDV’s)\textsuperscript{110} approximately 80 - 100nm in diameter, which form in the trophozoite stage and appear in low numbers. EDV’s contain PfEMP1 and PfEMP3 on their surface\textsuperscript{98,111} and fuse with the erythrocyte membrane\textsuperscript{110}, suggesting EDVs may be involved in the delivery of integral membrane protein cargo to the erythrocyte membrane\textsuperscript{19}. Similar structures, described as caveolae, have been observed in trophozoite stages of \textit{P. falciparum}\textsuperscript{112} and other \textit{Plasmodium} species, \textit{P.}
vivax and P. cynomolgi\textsuperscript{113}, indicating they may share a conserved function\textsuperscript{112,114}.

In addition to inducing new structures within the host erythrocyte, an increase in erythrocyte permeability also occurs following infection\textsuperscript{115}. This stems from the parasite inducing new permeation pathways (NPPs) in the erythrocyte to facilitate the transport of small molecules such as monosaccharide sugars, amino acids, peptides, nucleosides, and monovalent inorganic anions and cations\textsuperscript{105,116} that the parasite requires as nutrients. Additionally, toxic waste bi-products such as lactate, are also excreted from the cell\textsuperscript{117}.

1.4.2 Protein export

In addition to the structural modifications the parasite imparts on the host erythrocyte, the parasite also exports its own proteins into the host cell. In order for \textit{Plasmodium} proteins to gain access to the host cell, they must traverse two separate membranes, the parasite plasma membrane and the PVM\textsuperscript{118}. The export pathway begins with co-translational targeting of parasite proteins to the endoplasmic reticulum (ER), followed by a classical eukaryotic secretion pathway to the parasite plasma membrane and release of proteins into the PV space\textsuperscript{119,120}.
1.4.2.1 Protein export and the role of the PEXEL/HT motif in protein export

Most parasite proteins destined for export contain a classical hydrophobic ER signal sequence at the N-terminal region, which facilitates co-translational insertion in the ER\textsuperscript{118,119,121}. Fusion of GFP to a classical signal sequence results in localisation of GFP to the PV\textsuperscript{120,121}, whereas fusion of GFP to sequences lacking such N-terminal signal sequence remain in the parasite cytosol. Varying forms of the signal sequence exist, however, some exported proteins such as glycoporphin-binding protein-130 and ring-expressed surface antigen (RESA), possess a recessed hydrophobic sequence, located more than 50 amino acids from the N-terminus. From the ER, proteins are trafficked via typical eukaryotic secretion to the parasite plasma membrane in secretory vesicles and fusion of these vesicles with the parasite plasma membrane releases soluble proteins into the PV. It is not known whether parasite proteins that contain a transmembrane domain(s), are inserted into the ER membrane or are maintained in a soluble form within trafficking vesicles and thus released directly into the PV space or inserted into the parasite plasma membrane, followed by extrusion\textsuperscript{122}.

Proteins destined for translocation beyond the parasite and into the host cell must be distinguished from those that localise to the PV or to other regions within the parasite that utilise the ER-secretory pathway. A highly conserved core set of five residues, RxLxE/Q/D, where x is any uncharged amino acid residue, was identified which specifically directs export. This motif has been termed the \textit{Plasmodium} EXport ELelement (PEXEL)\textsuperscript{123} or Host-Targeting Signal (HT)\textsuperscript{124}. Chimeric constructs comprising the N-terminal region of three soluble exported proteins containing the ER signal sequence
and PEXEL motif fused to GFP, were shown to be trafficked beyond the PVM into the erythrocyte cytosol, indicating these N-terminal regions are sufficient for protein export\textsuperscript{123}.

The PEXEL motif is proteolytically cleavage in the ER by the aspartic acid protease Plasmepsin V\textsuperscript{125-128,129}. Since Plasmepsin V processing occurs in the ER, it suggests that sorting of cargo to be exported occurs early in the secretory pathway\textsuperscript{125,127,128,130}. Cleavage of the PEXEL motif occurs between residue three and four, after the leucine, resulting in a protein with a new N-terminus (xE/Q/D)\textsuperscript{129-131}, which is then N-acetylated (Ac-XE/Q/D)\textsuperscript{129}. Mutations of the conserved arginine (R), leucine (L), or R/L/glutamine (E) to Alanine (A) abrogates N-terminal protein cleavage\textsuperscript{125}, leading to retention of the protein in the ER\textsuperscript{127,131}, as well as accumulation in the PV\textsuperscript{123,124}. In contrast, mutation of the conserved fifth amino acid (E/Q/D) alone does not inhibit PEXEL cleavage or N-acetylation, indicating this residue is not essential for PEXEL processing\textsuperscript{125}. However, while not important for cleavage, amino acid residues 4 and 5 appear to be important for efficient protein trafficking and export, signifying the N-terminus of the mature PEXEL is important for directing correct export to the host cell\textsuperscript{127,131-134}.

\textbf{1.4.2.2 PEXEL containing exported proteins}

The discovery of the highly conserved PEXEL/HT motif in malaria parasites has enabled prediction of the proteins that are exported in \textit{Plasmodium} species, including those that infect rodents, birds, apes and humans\textsuperscript{123,124,135,136}. The prediction algorithm developed by Sargeant et al (2006)\textsuperscript{135}, termed ExportPred, was used to predict the number of exported
proteins in the *Plasmodium* genus based on the presence of a signal sequence and a PEXEL motif. Of ~5000 *P. falciparum* proteins, 396 were predicted to be exported, and many of these proteins contain one or two predicted transmembrane domains. Variation in the PEXEL motif has also been considered, including an export motif containing an arginine instead of a lysine (KxLxE/Q/D), or a relaxed PEXEL motif that includes an insertion of an amino acid between residues 4 and 5 (RxLxxE/Q/D)\textsuperscript{123,124}. However, only RxLxE and a relaxed PEXEL motif (RxLxxE/Q/D) have been shown to be proteolytically cleaved by Plasmepsin V, whereas the putative PEXEL motif KxLxE/Q/D is not\textsuperscript{134}. Based on further advances in the ExportPred (v2) algorithm, combined with gene modeling and annotation, the *P. falciparum* PEXEL exportome is now predicted to comprise of 463 proteins\textsuperscript{134}.

The PEXEL motif is also conserved amongst the *Plasmodium* species\textsuperscript{135} being largely similar across eight different *Plasmodium* species examined to date (*P. falciparum*, *P. reichenowi*, *P. vivax*, *P. knowlesi*, *P. gallinaceum*, *P. chabaudi*, *P. yoelii*, and *P. berghei*)\textsuperscript{109,136}. The most prominent difference in the PEXEL motif between the species is at the positively charged amino acid at position 1 of the PEXEL motif from arginine (R) to lysine (K)\textsuperscript{136}.

Based on the *P. falciparum* PEXEL motif algorithm, fewer PEXEL-positive proteins are present in non-\textit{falciparum} species (Table 1.1). For example, *P. berghei* is predicted to export only around 75 PEXEL positive proteins into the host cell\textsuperscript{137}. This is around six times smaller than the *P. falciparum* exportome. The large *P. falciparum* exportome, compared to other species is partly because of radiation and expansion of gene families in *P. falciparum* including those containing DnaJ domains\textsuperscript{138}, the *Plasmodium* helical interspersed subtelomeric (PHIST) family\textsuperscript{135} and Stevors/Rifins.
Approximately 75% of all the *P. falciparum* exported proteins belong to these protein families, whilst the remaining proteins (~200) are unrelated\(^{49,135,139,140}\). Also unique to *P. falciparum*, is the increased number of exported proteins required for the export of PfEMP1 to the parasite infected erythrocyte surface\(^{141}\). Multiple proteins have been identified (eg: SBP1, PFB0106c, MAL7P1.172, PF14_0758, and PF13_0076), that are required for the correct trafficking of PfEMP1 to its final destination at the erythrocyte surface\(^{140,142,143}\).

Regardless of the size of their respective exportomes and differences in the number of PEXEL proteins, the presence of a functionally conserved PEXEL motif among *Plasmodium* species\(^{109,123,135,144,145}\), suggests that protein trafficking and host-cell remodeling is conserved among the *Plasmodium* spp. Further supporting this is the observation that exported proteins are present on the infected erythrocyte surface, as well as in vesicular-like membranous structures in erythrocytes infected with *P. falciparum* and other species such as *P. berghei*\(^{95,96,109,146,147}\).

**Table 1.1: Comparison of the *P. falciparum* & *P. berghei* exportome**

<table>
<thead>
<tr>
<th></th>
<th><em>P. falciparum</em></th>
<th><em>P. berghei</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PEXEL proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Protein families</td>
<td>&gt;26 (eg: Rifin, Stevor)</td>
<td>&gt;5 (eg: Fam-b)</td>
</tr>
<tr>
<td>- Unique protein</td>
<td>108 (eg: KAHRP)</td>
<td>&gt;75 (eg: SMAC)</td>
</tr>
<tr>
<td><strong>PNEP proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Protein families</td>
<td>&gt;1 (eg: PfEMP1)</td>
<td>&gt;1 (eg: BirRs)</td>
</tr>
<tr>
<td>- Unique protein</td>
<td>&gt;15 (eg: SBP1)</td>
<td>&gt;20</td>
</tr>
<tr>
<td><strong>Total exported proteins</strong></td>
<td>~463</td>
<td>~350</td>
</tr>
</tbody>
</table>

*1 PEXEL proteins that are members of protein families
*2 PEXEL proteins that are unique and which do not belong to a protein family
*3 PNEP proteins that are members of protein families
*4 PNEP proteins that are unique and which do not belong to a protein family
1.4.2.3 PEXEL Negative Exported Proteins (PNEPs)

Whilst the identification of the PEXEL motif has been a valuable prediction tool for exported proteins, there are known exported proteins that lack the classical PEXEL motif\textsuperscript{123}, which are termed PEXEL negative exported proteins (PNEPs). This class of exported proteins contain no distinct structural features or a common motif that can be used to predict their export\textsuperscript{122,148}. Initially only a handful of exported proteins were identified as PNEPS, including SBP-1\textsuperscript{149}, membrane-associated histidine-rich protein 1 (MAHRP-1\textsuperscript{150}), MAHRP2\textsuperscript{100} and ring exported proteins (REX1)\textsuperscript{151} and REX2\textsuperscript{152}. These proteins localise to Maurer’s clefts\textsuperscript{149-152} or tethers\textsuperscript{100}, and some contain a single internal hydrophobic stretch\textsuperscript{148,152}. However by searching for proteins with a similar expression profile to the known PNEPS, 10 novel candidates were recently identified, indicating they are more numerous and diverse than initially anticipated\textsuperscript{139} and might play a more significant role in host cell remodeling\textsuperscript{122}. Protein unfolding of PNEPs is also required for their translocation, which further suggests that these proteins follow the same pathway as PEXEL positive proteins\textsuperscript{139}. While the rodent \textit{Plasmodium} species contain fewer PEXEL proteins than in \textit{P. falciparum}, they appear to export a larger repertoire of PNEPs\textsuperscript{109}, (such as erythrocyte membrane associated protein 1. For example, the \textit{P. berghei} genome encodes more than 120 PNEPs\textsuperscript{109}, with \~{}100 PNEPs assigned to a family of proteins called the BIR proteins, belonging to the overarching \textit{Plasmodium} interspersed repeat (PIR) superfamily, which can be found in other rodent \textit{Plasmodium} species such as \textit{P. yoelli} (YIR)\textsuperscript{153} or \textit{P. chabaudi} (CIR)\textsuperscript{154} as well as \textit{P. vivax} (VIR)\textsuperscript{155}. In addition, the Pb-fam-a protein family in \textit{P. berghei} contains 23 members, along with other unique PNEPs in this species, which
do not belong to either of these families. Thus, combined with PEXEL positive proteins, the *P. berghei* putative exportome, including PEXEL positive and PNEP exported proteins, comprises approximately 350 proteins.

### 1.5 Protein translocation across the PVM

The discovery of a common PEXEL motif between most exported proteins indicated that a common mechanism is used by the parasite to traffic and export proteins across the parasite membrane and PVM. Whether the same can be said of PNEPs is unknown as is whether they use the same export pathway to PEXEL proteins or trafficked separately.

It has been hypothesised that for proteins to cross the PVM, either vesicular mediated transport is utilised by the parasite, although no evidence exists for this pathway, or alternatively, a translocon is present at the PVM. If parasite proteins cross the PVM via a translocon, it is anticipated that the proteins arriving in the PV are in an unfolded state to be competent for export and that energy is also required to cross this membrane. Support for the translocon hypothesis came from experiments showing that, exported proteins fused to mouse dihydrofolate reductase (mDHFR) that can be blocked from unfolding using the drug WR99210, remain trapped in the PV. Moreover, depletion of ATP from the erythrocyte cytosol arrests protein transport in the PV.

To understand how *Plasmodium* parasites traffic proteins across the PVM, and to help identify a possible translocon, other translocation systems that
exist in nature which coordinate the delivery of multiple distinctive classes of protein substrates across various membranes to a range of destinations\textsuperscript{158,159}, have been compared with the anticipation of finding similarities (e.g. chloroplast, mitochondria and bacteria secretion systems). All these translocons comprise of three core features: receptor(s) to recognise the cargo to be translocated, the pore-forming component that provides a portal for proteins to cross the membrane and a motor\textsuperscript{158-160} to provide energy for the translocation process. In addition, translocons also comprise of other structural proteins to hold the complex in a particular conformation, regulatory proteins that may serve diverse roles, such as aiding the assembly of the translocon or to maintain in an open competent or closed state.

Protein substrates are directed to the translocon pore by the receptor components associated with the translocon complex. Receptors may be anchored to the membrane via a transmembrane domain or bind cargo in the cytosol and then peripherally associate with the membrane through interaction with other translocon components. Receptor proteins vary in size, structure and in their degree of conservation\textsuperscript{161}.

The translocon pore is an aqueous channel formed by membrane-embedded proteins which proteins assume highly stable structure\textsuperscript{162}. The manner in which proteins insert into the membrane to form the channel can vary. In mitochondria, the translocon at the outer membrane of mitochondria (TOM) pore-forming component, TOM40, forms a $\beta$–barrel structure through the membrane, with several TOM40 molecules forming 2-3 pores\textsuperscript{163-166}. In contrast, the main pore-forming component of the translocon at the inner
membrane of chloroplasts (TIC), Tic110, is mostly a \(\alpha\)-helical protein\(^{167}\). Both channels are cation selective and are between 17-25 Å in size.

Associated with the translocon is a component that serves as the energy source to aid translocation. The motor component may be bound on the trans or cis side of the membrane to pull (e.g., hsp70 of the ER in mitochondria\(^{168}\)) or push (e.g., SecA at the bacterial plasma membrane\(^{169}\)) across the membrane. Both of these examples are ATP-driven, however GTP-driven motors also exist.

Other components that contribute to protein translocation include chaperones. These prevent protein cargo from misfolding, aggregating or interacting with other cellular proteins prior to or after translocation\(^{158}\), and commonly depend on adenosine triphosphate (ATP) or guanosine triphosphate (GTP) hydrolysis for their activity\(^{158}\). Chaperone proteins can play two roles in protein translocation: Firstly, as most membrane systems can only transport proteins that are at least partially unfolded, chaperones help to maintain substrates in a translocation-competent state\(^{158,170,171}\). Secondly, in some translocons, chaperones may also provide energy for translocation, such as Toc159, which is not only a receptor in the translocases of the outer membrane of chloroplasts (TOC), it also acts as a GTP-driven motor that threads pre-proteins into the Toc-channel of chloroplasts\(^{172}\).

1.5.1 The Plasmodium Translocon of Exported Proteins (PTEX)

Although it was hypothesised that proteins cross the PVM by passing through a translocon, sequence homology searches for proteins that are
orthologues of components of known translocon systems described in section 1.5, failed to identify candidates\textsuperscript{1}. However, proteomic analysis of schizonts stage parasites revealed a lipid-raft-like detergent resistant membrane preparation that was enriched for proteins known to localise at the PVM. Thus by using specific criteria, and performing biochemical and cell biology analysis, de Koning-Ward \textit{et al} (2009)\textsuperscript{1} were able to identify the malaria protein export machinery termed \textit{Plasmodium} Translocon of Exported Proteins (PTEX), which comprises of five putative translocon components. These include HSP101 (heat shock protein 101, a AAA+ ATPase), EXP2 (a previously identified PVM protein\textsuperscript{173,174}, TRX2 (a thioredoxin-like protein)\textsuperscript{175}, and PTEX150 and PTEX88, so named based on their migration size by SDS-PAGE. Each of these five components is unique to and conserved within the \textit{Plasmodium} genus.

In initial studies of PTEX, a series of reagents were generated to characterise the complex, including specific antibodies recognising \textit{P. falciparum} PTEX150 (PfPTEX150), \textit{P. falciparum} HSP101 (PfHSP101) and \textit{P. falciparum} EXP2 (PfEXP2), as well as the generation of two transgenic \textit{P. falciparum} parasite lines in the strain 3D7, termed 3D7-150HA and 3D7-101HA, where the endogenous genes were modified to include a triple hemagglutinin (HA) epitope tag at their carboxyl termini\textsuperscript{1}. Expression profiles of HSP101 and PEX150 showed that both proteins are strongly expressed in late schizogony and remain at high levels through the 48 hour blood-stage cycle. Immunofluorescence analysis (IFA) showed that PTEX150, HSP101 and EXP2 co-localize and are found in discrete foci at the PVM in ring-stage parasites. In the schizont stages, these three proteins localise towards the
apical end of merozoites, with EXP2, shown by immuno-electron microscopy to localise to the dense granules\textsuperscript{1,176}.

Of the five PTEX components, none are predicted to form a $\beta$-barrel like structure, nor do any possess classic transmembrane domains\textsuperscript{1}. Thus no obvious candidates for the membrane-spanning, pore-forming component were identified. However, EXP2 is the PTEX component most tightly associated with the PVM, being resistant to carbonate extraction\textsuperscript{174}, requiring detergent to extract it from the membrane\textsuperscript{1,176}. EXP2 is predicted to have structural similarities with the bacterial hemolysins (pore-forming toxin) Cytolysin A from \textit{Escherichia coli}\textsuperscript{1,177,178}, an $\alpha$-helical protein which forms oligomers and insert into the target membranes via a $\beta$-tongue hydrophobic hairpin\textsuperscript{179}.

Analysis of the predicted structure of \textit{P. falciparum} HSP101 suggests that due to the conserved fold of this class of HSP100-AAA\textsuperscript{-}-ATPase family\textsuperscript{180} of chaperones, it forms a ring-shaped hexamer. Members of this family direct proteins through the central pore of their ring, using energy derived from ATP hydrolysis to unfold protein cargo\textsuperscript{156,176}. It is predicted, therefore, that HSP101 serves as the PTEX motor.

The function of PTEX150 is difficult to predict, since it does not show any homology to any other known proteins\textsuperscript{1}. As PTEX150 interacts tightly with HSP101 and EXP2, it may play a structural role in the complex, or may serve as a receptor\textsuperscript{176}. Overall, PTEX is a stable macromolecule complex of >1230kDa\textsuperscript{176}. EXP2 has the strongest vacuolar membrane association and forms the tightest association with PTEX150, which in turn binds more tightly to HSP101\textsuperscript{176}.
The two other components that were identified as part of PTEX but which remain to be validated, include TRX2 and PTEX88\(^1\). TRX2 is a 19-kDa protein and a member of the thioredoxin protein family, containing the typical WCQAC motif in its active site, which can be reduced by thioredoxin reductase\(^{181,182}\). Interestingly, a GFP-TRX2 fusion protein had previously been localised to the mitochondrion\(^{183,184}\), although intriguingly TRX2 lacks a mitochondrial targeting sequence\(^1,181\). Subsequent independent localisation studies reveal that *P. falciparum* TRX2 localises to the PV\(^{185-187}\), as well as to an unknown parasite organelle\(^{187}\). PTEX88 on the other hand, has no homology to other known proteins and no unique motifs to predict its role, and thus nothing is known about its functional role in PTEX. To date, no reagents (antibodies or epitope tagged chimeras) have been developed to facilitate the study of PTEX88. To date, the proposed functions of PTEX88 and TRX2 include a scaffolding role, recognition of cargo, or a regulatory role in the PTEX complex\(^1\).

With this information in hand, the current model of protein translocation in the infected erythrocyte is that proteins destined for export are trafficked to and deposited into the vacuolar space via a classical eukaryotic secretion pathway, and are then recognised by PTEX by a yet unknown receptor (Figure 1.2). HSP101 interacts with the substrate proteins to maintain them in an export competent state (which may also require the co-operation with other chaperone proteins) and provides the energy that enables proteins to be translocated across the PVM via the EXP2 pore. Once on the host cytosolic side, the translocated proteins are refolded and/or further trafficked, which likely includes further interactions with chaperones in the infected erythrocyte\(^1\). *Plasmodium* chaperones mediating these processes are yet to
be identified, however in immunoprecipitation experiments, a host derived HSP70\(^1\) was found to interact with PTEX and may play a role in these processes. Additionally, parasite derived exported hsp40 and hsp70-x may also be involved\(^{188}\). Given the number of proteins that comprise translocons in other organisms and organelles\(^{189}\), additional components of the PTEX may yet remain to be identified.

Figure 1.2: Schematic model of PTEX.

Expanded view of the PTEX complex at the PVM. Magnified schematic depicts EXP2 is the membrane spanning pore due to its strong membrane association. EXP2 is believed to interact most tightly with PTEX150, which in turn binds HSP101. PTEX88 and TRX2 are associated with PTEX, yet their composition within PTEX is unknown.
Although PTEX has been shown to interact with PEXEL exported proteins based on immunoprecipitation experiments of three exported proteins\(^1\), to date the evidence for the role of PTEX in protein export is circumstantial. A recent study using de-convolution microscopy and three-dimensional structures illumination microscopy demonstrated that PTEX components are present at the parasite periphery, and that they partially co-localise with a PEXEL containing exported protein, termed ring-infected erythrocyte surface antigen (RESA)\(^{176,190}\). Nonetheless, whilst this provides further support for PTEXs role in export, it is not conclusive proof. Furthermore the interaction of PNEPs with this complex is yet to be observed. It is therefore unclear whether the translocation of both subsets of exported proteins into the erythrocyte host proceeds via the PTEX complex, or whether multiple export pathways may be utilised by the parasite to export proteins containing the PEXEL motif and PNEPs\(^{122,145}\). However, since PEXEL positive proteins are cleaved, after which they possess no discernable motif (PNEPs also possess no discernable motif), it may be that a singular export pathway may accommodate both types of exported proteins\(^1,122,176\). Either way, definitive proof that PTEX is responsible for the export of PEXEL positive or PNEP exported proteins is still required.

1.6 Project aims and hypothesis

The overarching hypothesis of this study is that PTEX is a functional protein export complex and that disruption of PTEX function will lead to the loss of protein export into the host erythrocyte. Given that exported proteins are essential for parasite survival and virulence, it is hypothesised that the
resulting disruption of PTEX function will have consequences on parasite growth and pathogenesis.

To test these hypothesis, this study aims to: (i) firstly, confirm that PTEX88 and TRX2 are in-deed PTEX components, (ii) knockout PTEX genes to prove essentiality of PTEX function and to identify which components are essential for parasite survival and (iii) functionally characterise the role of the PTEX machinery in parasite protein export, parasite growth and virulence.

To further characterise the individual components that make up PTEX and determine their role in protein export, the model rodent malaria parasite, *P. berghei*, will be used in this study. Since PEXEL containing proteins are present in *P. berghei*, and host cell remodelling is conserved, as well as conservation of the genes encoding the PTEX between the species¹⁹¹, it is therefore likely that the mechanism of protein export is also conserved and it is likely that *P. berghei* is an applicable model. *P. berghei*, is more amenable to genetic manipulation than *P. falciparum*, and given that a rodent model is available for *P. berghei*, the *in vivo* consequences of blocking PTEX function can be assessed, including determining the contribution of PTEX to parasite virulence and growth. This is particularly important for validating whether PTEX could serve as a good anti-malarial drug target.
Chapter 2

Materials and Methods
1.7 Materials

Reagents and equipment used in this research was supplied from the following manufacturers:

- Abbott       Illinois, U.S.A
- Abcam       Cambridge, UK
- Amresco       Solon, OH, USA
- Axygen       Union City, CA, USA
- Becton Dickinson Franklin Lakes, NJ, USA
- Bioline       London, UK
- Bio-Rad Hercules, CA, USA
- BOC Australia
- Dako Denmark
- Fermentas Ontario, Canada
- Fisher Biotech Waltham, MA, USA
- Fluka Biochemika Buchs, Switzerland
- General Electric Healthcare (GE) Fairfield, CT, USA
- Genesearch QL, Australia
- HemoCue Ångelholm, SWEDEN
- Ilford Switzerland.
- Integrated Sciences Georgia, USA
- Interpath Vic, Australia
- Invitrogen Groningen, The Netherlands
- John Morris Scientific NSW, Australia
- Lonza Basel Switzerland
- Merck/Calbiochem San Diego, CA, USA
- Millipore Billerica, MA, USA
- Miltenyi Biotech Auburn, CA, USA
- New England Biolabs Ipswich, MA, USA
- Novagen Madison, WI, USA
- Pacific Laboratories Redmond, WA, USA
- Promega Fitchburg, WI, USA
- Proscitech QL, Australia
- Provet QL, Australia
- Roche Molecular Biochemicals Indianapolis, IN, USA
- Qiagen Mel, Australia
- Sigma - Aldrich Saint Louis, MO, USA
- Thermo Fisher Scientific Rochester, NY, USA
- VWR Radnor, PA, USA
- Whatman Kent, UK
1.8 Methods

1.8.1 Ethics statement

All experiments involving rodents were performed in strict accordance with the recommendations of the Australian Government and National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes. The protocols were approved by the Animal Welfare Committee at Deakin University (Approval No.: AWC A97/10, A98/2010, G37/2014).

1.8.2 DNA and protein sequence analysis

*Plasmodium* gene sequences were extracted from the *Plasmodium* genome Resource database (PlasmoDB)\(^{191}\). *In silico* restriction enzyme digests and sequence alignments were performed using SerialCloner version 2.0 (Serialbasics).

1.8.3 Oligonucleotide Primers

All oligonucleotides used in this project were obtained from Geneworks and are all listed in Table 2.1.

1.8.4 DNA applications

1.8.4.1 DNA preparation

Small or large-scale plasmid preparations were made from 2 ml or 400 ml overnight bacterial cultures, respectively, and grown under agitation (200 rpm) at 37 °C in Luria broth (Amresco) containing 100 µg/ml ampicillin. The DNA was extracted and purified using an Invitrogen mini-prep kit or midi-prep kit as per the manufacturers instructions.
1.8.4.2 Polymerase Chain Reaction (PCR) amplification

DNA was amplified by PCR using plasmid or parasite genomic DNA (gDNA) as template. Each 20 μl reaction contained 200 μM dNTP, 0.5 U Taq (NEB), 200 nM of each oligonucleotides and 10 ng plasmid or 100 ng gDNA template in 1 × PCR amplification buffer (NEB). Typical cycling conditions were an initial 2 min cycle of 95 °C, followed by 25 cycles of 95 °C (denaturation) for 1 min, 50 °C (annealing) for 1 min and 68 °C (extension) for 1-2 min (according to the expected product size). This was followed by one cycle at 72 °C for 5 min, after which the sample was held at 4 °C. Colony screening by PCR was performed using the same conditions except the bacterial colony was used as template DNA. Amplified DNA was then purified using the PureLink™ PCR Purification Kit (Invitrogen) and eluted with TE (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA yield was measured using the NanoVue Spectrophotometer according to the manufacturers instructions (General Electric).

1.8.4.3 DNA digestion

DNA (100 ng - 5 μg) was digested with restriction enzymes supplied by New England Biolabs, according to the manufacturers instructions. To de-phosphorylate the cloning vectors, 1 U of shrimp alkaline phosphatase (Fermentas) was added to the vector and left to further incubate at 37 °C for 1 hr.

1.8.4.4 DNA ligation

Vector and insert DNA were ligated with 1-5 U of T4DNA ligase in 1 × ligase buffer (Fermentas) at a 2:1 insert to vector ratio and incubated at room temperature for 15 min. A control ligation containing only vector DNA and no
insert was performed in conjunction to assess the frequency of self-ligation of the plasmid vector.

1.8.4.5 Bacterial transformation

DNA was transformed into *Escherichia coli* (*E. coli*) XL-10 Gold cells using the heat-shock method. For this, up to 500 ng of DNA was incubated for 2 min on ice with a 200 μl aliquot of cells prepared according to “*Current Protocols in Molecular Biology* library”\(^{192}\) followed by incubation at 42 °C for 45 sec. Immediately afterwards, 1 ml Luria broth (Amresco) was added to the cells then incubated under agitation of 200 rpm for 45 min at 37 °C to allow expression of the ampicillin resistance cassette. Transformed cells were spread onto Luria agar (Amresco) containing 100 μg/ml ampicillin and incubated overnight at 37 °C.

1.8.4.6 DNA sequencing

DNA was sequenced by the Australian Genome Research Facility Ltd (AGRF) at The Walter and Eliza Hall Institute (WEHI) using the Big Dye Termination sequencing protocol with 20 pmol of either the appropriate forward or reverse oligonucleotide and 500-1500 ng template DNA used per reaction.

1.8.4.7 Agarose gel electrophoresis

DNA was routinely separated on 0.7-2.0 % (w/v) horizontal agarose gels using a Tris-borate-EDTA (TBE) buffer system (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA). Loading buffer (0.01 % (w/v) bromophenol blue, 10 mM EDTA and 10 % (w/v) glycerol) was added to sample prior to loading. A 1 kb molecular marker (Invitrogen) was included on each run. Gels were electrophoresed between 80-110 V for 30 min to 3 hrs. Agarose gels
contained SYBR Safe (Invitrogen) at a concentration of 1:10,000 for DNA visualization on a ChemiDoc XRS (Bio-Rad) UV transilluminator.

1.8.4.8 Southern blot hybridisation

1.8.4.8.1 Preparation of membrane

After electrophoresis, agarose gels containing digested gDNA were depurinated in 0.125 M hydrochloric acid solution for 20 min, followed by soaking in denaturation solution (0.5 M sodium hydroxide and 1.5 M sodium chloride) for 30 min. The gel was then neutralised in 0.5 M Tris and 1.5 M sodium chloride (pH 8.0) for 30 min and DNA subsequently transferred in the neutralisation buffer onto Hybond-N membrane (General Electric) via capillary action overnight. Following transfer, the membrane was baked in an incubator at 80 °C for 2 hrs to fix the DNA to the membrane. The membrane was rinsed in Milli-Q water, air-dried at room temp and stored at 4 °C until hybridization.

1.8.4.8.2 Digoxigenin labeled DNA probes

Probes used for Southern blot hybridization were amplified using the DIG-PCR Probe synthesis kit (Roche). Each 50 μl reaction mix contained 35 μM DIG-dUTP (PCR DIG Probe synthesis Mix) with 200 μM each dNTP, along with 200 nM each forward and reverse oligonucleotide and template DNA at a concentration of 1-50 ng. HotStartTaq DNA polymerase (Qiagen) was used at a final concentration of 2.5 U with the final MgCl₂ concentration at 2.0 mM in the 1 × PCR buffer (Qiagen). PCR cyclic conditions were an initial heat activation step at 95 °C for 15 min, followed by 35 cycles of 94 °C (denaturation) for 30 sec, 60 °C (annealing) for 30 sec and 68 °C (extension)
for 4 min. This was followed by a 72 °C incubation step for 20 min. After amplification, samples were stored at –20 °C.

1.8.4.8.3 Hybridisation

The membrane containing DNA was pre-hybridised with 10 ml DIG Easy-Hyb solution (Roche) at 37 °C for at least 1 hr. This was followed by hybridisation with the DIG-labeled probe (~30 ng/ml) in 10 ml Easy-Hyb solution at 37 °C overnight.

1.8.4.8.4 Detection

Following hybridization the membrane was treated to a series of washes at room temperature with slow rocking. An initial rinse with 2×SSC for 5 min was then followed by equilibration in wash buffer solution of 0.1M maleic acid (Sigma-Aldrich), 0.15 M sodium chloride and 0.3% (v/v) Tween-20 (Sigma) for 1 min. Incubation of the membrane for 30 min in 1% (v/v) blocking solution (Roche) was then followed with Digoxigenin-AP antibody (Roche) binding at a dilution of 1:10,000 in blocking solution for 1 hr. The membrane was then washed in wash buffer for 30 min and equilibrated in detection buffer containing 100 mM Tris-HCL (pH 9.5) (Invitrogen) and 100 mM NaCl for 2 min. The membrane was drip dried then transferred to a hybridization bag where it was drenched in pre-warmed CSPD ready-to use (Roche) and immediately covered from light and left to incubate for 5 min. The membrane was transferred to a cassette and exposed to X-ray film (GE) for various exposure times (generally 5, 10, 30 min depending on signal strength). The films were then developed using Ilford Ilfosol developer at a ratio of 1:9 and fixed with Ilford fixer at a ratio of 1:14.
1.8.4.8.5 Stripping probes from membrane

Probes were stripped from the membrane by rinsing the membrane in Milli-Q water for 1 min followed by two separate washes with freshly prepared 0.2M NaOH containing 0.1% (v/v) SDS. The membrane was then rinsed in 2 \( \times \) SSC for 5 min and stored at 4 °C.

1.8.5 Plasmid constructs

1.8.5.1 Epitope tagging

To create the vectors that were used to epitope tag the endogenous \( P. \) \( berghei \) ANKA (Pb) PTEX genes, the following cloning procedures were performed. First \( \sim 2 \) kb immediately upstream of the stop codon of PbPTEX150 (which would ultimately serve as the targeting region for homologous recombination into the PbTEX150 endogenous locus) was PCR-amplified from \( P. \) \( berghei \) ANKA genomic DNA (gDNA) in roughly two equal parts using the oligonucleotides O62/O63 (5' targeting region) and O64/O65 (3' targeting region) (Refer to Table 2.1 for oligonucleotide sequences). By sewing the two resulting PCR products together with O62/O65, this created a SpeI site between the two adjacent 5' and 3' sequences that would later be used to linearize the DNA prior to transfection. The sewn PCR product was then digested with NotI and PstI and cloned in place of the \( P. \) \( falciparum \) PTEX150 in pTEX150-HA/Str 3'1. Subsequent digestion with NotI and EcoRV excised the PbTEX150-HA/Str sequence together with the downstream \( P. \) \( berghei \) DHFR-TS 3' UTR and, after treatment with Klenow, was cloned into the pB3 vector (Waters \( et \) \( al. \), 1997)\(^{193} \) that had been digested with ClaI and treated with Klenow. As the backbone of this resulting vector contained a SpeI site, this was removed by digestion with Nael and EcoRV that flank this
site, followed by re-ligation of the plasmid, to give the final epitope tagging construct termed pB3-150-HA/Str-DT3'. From this vector all subsequent P. berghei PTEX tagging constructs were made by replacement of the PbTEX150 5' and 3' targeting regions using SacII/Spel and SpeI/AvrII, respectively, with the targeting sequences of HSP101 (amplified using O46/O33 and O34/O35), TRX2 (O36/O51 and O52/O53), PTEX88 (O54/O55 and O56/O57) and EXP2 (O58/O59 and O60/O61). Prior to transfection into P. berghei ANKA parasites, all targeting constructs were linearized with Spel to drive integration into the endogenous PTEX locus.

1.8.5.2 PTEX gene deletion

For targeted gene deletion of the PTEX genes, fragments of the 5' UTR and 3' UTR that would serve as targeting regions to drive integration into the endogenous locus were PCR-amplified from P. berghei ANKA gDNA using gene-specific primers. The 5' UTR homologous sequences were digested with Apal/Sacl (or HindIII/Sacl in the case of the PTEX88 5' UTR), with the SacII site filled in with Klenow, and cloned into the pB3 vector that was digested with ClaI, treated with Klenow and then digested with Apal. The 3' UTR targeting sequences of HSP101 (digested with EcoRV/NotI), EXP2 (EcoRV/SmaI), PTEX88 (EcoRV/EcoRI) and TRX2 (EcoRI/Spel) were cloned into the corresponding sites of pB3 containing the respective 5' UTR or, in the case of EXP2 and TRX2 3' UTRs, into the Spel site that had been treated with Klenow. The resulting constructs were linearized with KpnI/NotI and used to transfect P. berghei ANKA parasites.
1.8.6 Protein applications

1.8.6.1 Protein expression

For large-scale protein expression, 10 ml Luria broth containing 100 µg/ml ampicillin was inoculated with *E. coli* transformants of interest and incubated at 37 °C overnight. From this overnight culture, 4 ml was removed and added to 400 ml Luria broth containing ampicillin in a 2 L pre-autoclaved flask. For small-scale protein expression, 2 ml overnight culture was inoculated into 50 ml Luria broth cell culture. Broths were incubated at 37 °C at 200 rpm until an optical density (OD) of 0.5 - 0.6 was reached. From this, 1 ml of culture was taken prior to induction of protein expression, representing the negative “un-induced” control. The remaining culture was induced for protein expression for 2 hrs with 1 mM Isopropyl-β-D-thiogalactoside (IPTG). After induction, a 1 ml sample was taken from the culture as a positive “induced” control. Both induced and un-induced control samples were stored at −20 °C until further analysis. The remaining cell culture was pelleted and the supernatant removed. The cell pellet was re-suspended in 15 ml 1 × native purification buffer (pH 8.0) containing 50 nM NaH$_2$PO$_4$ and 0.5 M NaCl. Lysozyme from chicken egg (1 mg/ml) (Invitrogen) was added along with 1 × tablet protease inhibitor cocktail (Roche) and incubated for 30 min at 4 °C under agitation. Cells were further lysed by 18 sonication bursts for 10 sec each at an amplitude of 60%.

1.8.6.2 Protein purification

Expressed protein was purified on Ni-NTA affinity resin according to the manufacturers instructions (Invitrogen). After an overnight binding incubation at 4 °C, three washes with 20 mM imidazole (Sigma) were performed,
followed by elution with 250 mM imidazole for 1 hr and then again overnight.

1.8.6.3 Protein electrophoresis

Infected rodent blood was depleted of leukocytes by passage through a CF11 column (Whatman) and then lysed using 0.02% saponin for 15 min on ice, followed by centrifugation at 14,000 rpm for 5 min. After three washes with ice-cold mouse tonicity (MT) PBS containing 1× complete protease inhibitors (Roche), and prior to electrophoresis, the parasite pellet was resuspended in 1× reducing sample buffer (4% (v/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue and 0.125 M Tris HCl (pH 6.8)). Protein samples were initially boiled at 100 °C for 5 min and micro-centrifuged at 14,000 rpm for 10 min. Sample supernatant (5-15 µl) and Dual Standards (Biorad) protein marker (5 µl) were loaded and fractioned by electrophoresis in either 4-12% gradient (Invitrogen) or 10-12% SDS-PAGE gel at 150 V for approximately 1 hr in 1× gel running buffer (25 mM Tris base, 192 mM Glycine, 0.1% (w/v) SDS (pH 8.3)). Gels were stained by soaking in Imperial Protein Stain (Thermo Scientific) for 30 min at room temperature. The gel was then soaked in de-stain at a final concentration of 50% (v/v) methanol with 10% (v/v) acetic acid for 45 min and left overnight at 4 °C in Milli-Q water.

1.8.6.4 Western blot analysis

SDS-PAGE polyacrylamide gels were prepared for protein transfer onto 0.45 µm PVDF membrane (Millipore) via the Bio-Rad wet transfer system in 1× Tris-glycine buffer (25 mM Tris base, 190 mM glycine, 0.1% (w/v) SDS). Transfer was performed at 100 V for 1 hr. The membrane was then incubated in 3% bovine serum albumin (BSA) / mouse - phosphate buffered saline (MT-PBS) block at 4 °C, followed by overnight incubation with the
antibody specific to the protein being detected diluted in 3% BSA/MT-PBS blocking solution. After three washes in 0.05% (v/v) Tween/MT-PBS, HRP conjugated secondaries (anti-rat, anti-rabbit or anti-mouse) were used at a 1/2000 dilution in a 3 hr incubation at 4 °C. Following a second series of three washes in MT-PBS, detection was performed using the SuperSignal enhanced chemiluminescence (Thermo Fischer Scientific) according to manufacturers' instructions, by exposure to X-ray film. Where applicable, band densities were measured using Image J software.

1.8.6.5 Protein immunoprecipitations

For immunoprecipitations (IP), *P. berghei* infected erythrocytes depleted of leukocytes or *P. falciparum* infected erythrocytes were lysed with 0.02% saponin/MT-PBS. Pelleted parasite material, were lysed with either IP lysis buffer (Pierce) or 0.5% Triton X-100 in PBS containing complete protease inhibitors (Roche). After a 30 min incubation on ice, the material was passed 10 times through a 29 G insulin needle, followed by centrifugation at 21,000 g for 10 min at 4 °C. The supernatant was affinity purified with either goat anti-HA antibodies coupled to magnetic beads (Miltenyi Biotec Australia) as per the manufacturer's instruction or with 150 μl anti-HA sepharose (Roche) for 8 hrs at 4 °C. Unbound material was collected and beads then washed four times with 1 ml 0.5% Triton X-100 in MT-PBS containing complete protease inhibitors (Roche) and once with 1 ml MT-PBS. Bound proteins were eluted with 50 μl 1 × reducing sample buffer and separated by SDS-PAGE.

For immuno-immune-detection, primary antibodies used were monoclonal mouse anti-HA (12CA5) (1:1000) (Roche), polyclonal rabbit anti-HSP101 (1/200 for *P. berghei*, 1/500 for *P. falciparum*), monoclonal mouse anti-
HSP101 (1/500), poly-clonal rabbit anti-PTEX150 (1:200 for *P. falciparum*), mono-clonal mouse anti-EXP2 (mAb 7.7, from J. McBride and described previously) (1/500 for *P. berghei* and 1:2000 for *P. falciparum*). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies (1:2000, Jackson IR).

### 1.8.6.6 Gel excision and Nano-LC-MS/MS

Protein bands were manually excised from separated SDS-PAGE gels and subjected to manual in-gel reduction, alkylation and tryptic digestion. The following methods were performed by Thomas Nebl (WEHI) for Nano-LC-MS/MS. All gel samples were reduced with 10 mM DTT for 30 min, alkylated for 30 min with 50 mM iodoacetic acid and digested with 375 ng trypsin (Promega) for 16 h at 37 °C. The extracted peptide solutions were then acidified (0.1% formic acid) and concentrated to approximately 10 μl by centrifugal lyophilization using a SpeedVac AES 1010 (Savant). Extracted peptides were injected and fractionated by nanoflow reversed-phase liquid chromatography on a nano- UHPLC system (Easy n-LC II, Thermo Fisher, USA) using a nanoAcquity C18 150 mm × 0.15 mm I.D. column (Waters, USA) developed with a linear 60 min gradient with a flow rate of 250 nl min⁻¹ from 100% solvent A (0.1% Formic acid in Milli-Q water) to 100% solvent B (0.1% Formic acid, 60% acetonitrile, (Thermo Fisher, USA) 40% Milli-Q water). The nano UHPLC was coupled online to an LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher, USA) for automated MS/MS. Up to five most intense ions per cycle were fragmented and analysed in the linear trap, with target ions already selected for MS/MS being dynamically excluded for 3 min.
Protein identification from the LC-MS/MS data were searched against a non-redundant protein decoy database comprising sequences from the latest version of LudwigNR (Human, Bovine, *P. falciparum* species), as well as their reverse sequences (249275 entries). Mass spectra peak lists were extracted using extract-msn as part of Bioworks 3.3.1 (Thermo Fisher Scientific) linked into Mascot Daemon (Matrix Science, UK). The parameters used to generate the peak lists for the LTQ Orbitrap were as follows: minimum mass 400; maximum mass 5000; grouping tolerance 0.01 Da; intermediate scans 1; minimum group count 1; 10 peaks minimum and total ion current of 100. Peak lists for each nano-LC-MS/MS run were used to search MASCOT v2.2.04 search algorithm (Matrix Science, UK) provided by the Australian Proteomics Computational Facility (http://www.apcf.edu.au). The search parameters consisted of carboxymethylation of cysteine as a fixed modification (+58 Da, for gel samples only), with variable modifications set for NH2-terminal acetylation (+42 Da) and oxidation of methionine (+16 Da). A precursor mass tolerance of 20 ppm, #13C defined as 1, fragment ion mass tolerance of ± 0.8 Da, and an allowance for up to three missed cleavages for tryptic searches was used.

1.8.6.7 Recombinant protein purification from maltose binding protein

Removal of the maltose binding protein (MBP) tag was performed using Factor Xa (FXa), which cleaves after the arginine residue at the Ile-(Glu or Asp)-Gly-Arg site. For this, 1 μg of FXa was used to digest 50 μg of substrate overnight at 4 °C. The sample was then concentrated by centrifuge column (Millipore). The proteins were then separated by 4-12% gradient SDS-PAGE gel electrophoresis and the band containing the desired protein fragment was
excised and used to immunise rabbits for antiserum production.

1.8.7  **Plasmodium applications**

1.8.7.1  **Infection of mice with *P. berghei*.**

The *P. berghei* CI15 of the ANKA strain was used for all mouse infection studies. Eight week old female donor Balb/c mice were infected with 200 µl of infected blood from liquid nitrogen stocks of *P. berghei* ANKA. To estimate the parasitemia of infected mice, thin blood smears were prepared by collection of a 2 µl drop of tail blood smeared onto a glass slide. These were fixed for 30 sec with methanol (Merck) and stained for 10 min with a 1/10 dilution of Giemsa stain (Merck) per manufacturers recommendation. The slide was rinsed with tap water and air-dried. The parasitemia was determined using a light microscope under immersion oil using the 100x objective counting the number of infected erythrocytes expressed as a percentage of uninfected erythrocytes. A combined total of 1000 cells were counted for each slide. To cryopreserve parasites, *P. berghei* infected whole blood was mixed with 30% (v/v) glycerol in phosphate buffered saline (PBS)) in a 1:1 ratio, and aliquoted into cryovials (Thermo Fisher Scientific) before being frozen in liquid N₂.

1.8.7.2  **Preparation of DNA for *P. berghei* transfection**

Between 15-20 µg of DNA was digested with 2-3 µl of the appropriate restriction enzyme (10-20 U/ul) overnight at 37 °C to linearise the transfection construct. Digested DNA was then separated in 1% agarose gel via electrophoresis. The appropriate DNA band was purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen) as per manufacturers instructions. DNA was concentrated by precipitation with 2.5 × vol 100%
ethanol, 1/10 × vol 3M NaAc (pH 5.2) and re-suspended in a final volume of 10 µl TE (~5 µg digested DNA).

1.8.7.3 Preparation of schizonts for *P. berghei* transfection

Female Balb/C mice (6-8 weeks old) were infected with 1 × 10⁶ PbANKA parasites intraperitoneally. When the parasitemia reached approximately 1-3%, blood was collected from the mouse between the hrs of 10:00 to 14:00 via cardiac puncture under anesthesia with 100% isoflurane (ProVet) or in an atmosphere of 100% carbon dioxide. Infected blood was spun down at 1500 rpm to remove the buffy coat and the erythrocytes were then added to 150 ml complete RPMI1640 culture medium containing L-glutamine (Invitrogen), supplemented with 5% sodium bicarbonate, and containing 25% (v/v) heat inactivated Fetal Bovine Serum (Bovogen). Parasites were incubated for 90 sec in an atmosphere of 5% oxygen, 1% carbon dioxide and 94% nitrogen to mimic the host gaseous environment then incubated 16 hrs at 36.5 °C. The following day, mature schizonts were purified by Nycodenz separation as previously described.

1.8.7.4 Transfection and drug selection

Prior to transfection, 100 µl of Amaxa Basic Parasite Nucleofector ® Solution (Lonza) was combined with ~10⁷ nycodenz purified schizonts and mixed gently with linearised DNA. The mixture was transferred to an electroporation cuvette (Lonza) and transfection was performed using an Amaxa® Nucleofector device set at program U33, to introduce the linearised DNA fragment into wild type parasites for integration into the genome. Once electroporated, 50 µl complete culture medium was added and the total volume (150 µl) was injected intravenously into the tail vein of a 6-8 week female Balb/c mouse.
Drug selection of transfected parasites commenced one day after the transfection procedure to allow parasites to complete one full developmental cycle. Pyrimethamine was administered via drinking water. Pyrimethamine was made at a concentration of 7 mg/ml; the pH was adjusted to 3.5 – 5.0 using 1 M HCL and then diluted 1/100 with tap water for a final concentration of 0.07 mg/ml. For secondary drug selection or where required, 16mg/kg WR99210, dissolved in DMSO and diluted with MT-PBS, was administered subcutaneously 1-3 days post infection.

1.8.7.5 Isolation of parasite DNA from whole blood.

To obtain parasite DNA from whole infected rodent blood collected by cardiac puncture, the leukocytes were first removed by chromatography using CF11 powder (GE). Eluted red blood cells were centrifuged at 3000 rpm and lysed in 0.15% (w/v) Saponin (Fluka BioChemika) in PBS (Amresco), after which free parasites were pelleted via micro-centrifuge at 14,000 rpm. For parasite genomic DNA isolation, parasites were resuspended in *P. berghei* parasite lysis buffer (50 mM Tris, 5 mM EDTA, and 1.0% (w/v) SDS, pH 8.0). RNase A (Invitrogen) was added to the sample at a final concentration of 100 μg/ml and incubated at 37 °C for 40 min after which Proteinase K (Invitrogen) was added at a concentration of 2 mg/ml and incubated at 37 °C for a further 45 min. The DNA was extracted three times using 1:1 phenol-chloroform mix (Sigma) and precipitated with 100% (v/v) ethanol following the standard procedure in ‘Current Protocols in Molecular Biology’; the pellet was then washed with 80% (v/v) ethanol, dried and suspended in 100 μl TE.
1.8.7.6 Cloning of transgenic parasites

To ensure that transgenic *P. berghei* parasite lines were pure and no wild type *P. berghei* ANKA parasites were present, a serial cloning procedure was performed. For this, six Balb/c mice were infected intravenously with limiting dilutions of parasite infected erythrocytes per mouse in 100 μl PBS, using a 26 G needle. Accurate total erythrocyte counts were obtained using a hemacytometer and clonal lines were confirmed by parasite genotyping.

1.8.7.7 Parasite growth curves

To assess whether transgenic parasites were affected in their growth and/or replication in comparison to wild type parasites, Balb/c mice were infected with $1 \times 10^5$ *P. berghei* ANKA wild type (n=14) or knockout clones (n=14). The parasitemia in blood smears made from each mouse was calculated on a daily basis from 3 days post infection. Mice were humanely culled when parasitemias exceeded 25%.

1.8.7.8 Parasite virulence analysis

As a measure of parasitic virulence, transgenic parasites were compared to wild type parasites in their ability to cause cerebral malaria in C57Bl/6 mice. Three groups of six C57Bl/6 female mice at six weeks of age, were infected with $1 \times 10^6$ PbANKA wild type or transgenic parasites. The parasitemias from each mouse was calculated from 3 days post infection and thereafter on a daily basis. Mice were checked every 4 hrs for cerebral malaria symptoms, which included ataxia, and the lack of the ability to self-right, from day 5 post infection. Mice were culled at a parasitemia exceeding 25% or at the sign of cerebral malaria symptoms.
1.8.7.9  **In vivo / in vitro growth assay**

Infected erythrocytes from three Balb/C mice were cultured for 16 hrs in complete RPMI medium supplemented with 25% FCS at 36.5 °C. *P. berghei* schizonts obtained from overnight cultures, were ruptured by mechanical action using a 29 G needle and syringe. Merozoites were purified by filtration through a 0.2 µm filter and then resuspended in 100 µl RPMI. These were then combined 1:1 with reticulocyte rich blood from phenylhydrazine (10 µL/g) pre-treated mice. Invasion was allowed to proceed for 30 min at 37 °C with during which time, the samples were vigorously shaken. The cells were then either cultured *in vitro* in complete RPMI or intravenously injected into naïve mice to establish synchronous mouse infections. *In vivo* parasites were monitored by blood smear at two hr intervals post invasion.

1.8.7.10  **Generation of semi-immune sera**

Semi-immune sera was generated from Balb/C mice which had been challenged with four consecutive malaria infections on a monthly basis by intraperitoneal injection of $10^4$ parasites. Each infection was cleared by administration of 0.07 mg/ml pyrimethamine via drinking water when the parasitemia reached no more than 5%. Ten days post final challenge, all mice were cardiac bled. For pre-immune sera, Balb/C mice that had never been exposed to a malaria infection were cardiac bled. Blood was allowed to clot overnight at 4 °C after which it was then centrifuged at 3,000 rpm to collect the pre-immune serum. The serum was heat inactivated at 65 °C and stored at -80 °C until use.

1.8.8  **Immunofluorescence analysis of *P. berghei***

Erythrocytes infected with *P. berghei* parasites were fixed with 4% (v/v)
paraformaldehyde and 0.0075-0.015% (v/v) glutaraldehyde in PBS for 30 min. After washing, cells were permeabilised with 0.25% Triton X-100 where indicated, for 10 min and then washed a further 3 times, before blocking with 1% BSA/MT-PBS (Sigma) for 30 min. Cells were labeled for 1 hr at room temperature with appropriate primary and secondary antibodies specific to the protein of interest, followed by three washes in 1xMT/PBS after each step. Cells were mounted in Vectashield containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (VectorLabs). Images were acquired with an Olympus IX70 microscope and processed using the Cell-X program. Further image processing was performed using Adobe Photoshop CS6.

1.8.9 Flow Cytometry

Flow cytometry was used to quantitate expression of *P. berghei* proteins on the surface of infected erythrocytes. Wild type and transgenic parasites were harvested from infected Balb/C via tail smear, and incubated in block (1% (w/v) casein sodium salt in RPMI buffer) for 30 min, followed by incubation for 1 hr in either pre-immune or hyper-immune sera diluted to 1/20. Samples were then centrifuged at 1,200 rpm for one minute to remove the supernatant followed by incubation in Goat anti-mouse IgG AlexaFluor 647 conjugate (Invitrogen) at a concentration of 1:2000 for 1 hr. Following two washes, cells were incubated for 5 min in Sybr safe (Invitrogen) in block at a dilution of 1:2000. Three washes completed the cell preparation before cell suspensions were analysed using a FACS Canto by BD. Settings were optimized per sample, as per manufacturers manual. All incubations were performed at ambient temperature.
1.8.10 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism version 6 (GraphPad Software - MacKiev). Results were analysed for statistical significance using a two-tailed student’s t-test using parametric distribution, One-way ANOVA with Tukey Post hoc analysis, and a log-rank (Mantel-Cox) test for survival analysis. A P value <0.05 was regarded as significant.
<table>
<thead>
<tr>
<th>Oligonucleotide reference</th>
<th>Target gene</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1 PbPTEX150</td>
<td>5' CGC GGA TCC GAT CTT TAG CAA TTA ATT CAA</td>
<td></td>
</tr>
<tr>
<td>O2 PbPTEX150</td>
<td>5' GGA CTG CAG AAG CTC ATC AGG GTG GAT GTA</td>
<td></td>
</tr>
<tr>
<td>O3 PbTRX2</td>
<td>5' CGG GGA TCC GAT CTT TTA AGA CCA CAC CTC</td>
<td></td>
</tr>
<tr>
<td>O4 PbTRX2</td>
<td>5' GGA CCG TAG TTA ATG TTT TTT AAT TAA TGC</td>
<td></td>
</tr>
<tr>
<td>O5 PbTRX2</td>
<td>5' CGG GGA TCC GAC TCC CAA TTA TCA CCA ATA G</td>
<td></td>
</tr>
<tr>
<td>O6 PbTRX2</td>
<td>5' CCG GAT CCG TAA ATG CTT TCT TCT CAA ATG TGA</td>
<td></td>
</tr>
<tr>
<td>O7 PbTRX2</td>
<td>5' TAG GCC CCT TCC TAG TAA AAT TAC GAC GTC</td>
<td></td>
</tr>
<tr>
<td>O8 PbTRX2</td>
<td>5' GCC GAA TAC CAG AGG AGT GTC AAG CGC TGG TAT</td>
<td></td>
</tr>
<tr>
<td>O9 PbTRX2</td>
<td>5' GAA AAC CCT AAA CAC ATT TGG TGC</td>
<td></td>
</tr>
<tr>
<td>O10 PbTRX2</td>
<td>5' GTG TTA GAA AAG AAG TTA ATA ATT C</td>
<td></td>
</tr>
<tr>
<td>O11 PbDHFR</td>
<td>5' ACT GTT ATA ATT ATG TGT CCT CTT C</td>
<td></td>
</tr>
<tr>
<td>O12 PbDHFR</td>
<td>5' GAT GTG TTA TGT GAT TAA TTA ATA C</td>
<td></td>
</tr>
<tr>
<td>O13 PbHSP101</td>
<td>5' TAG GAC CGG CCT TTA TAG ATG GTG CCT CTT TAA A</td>
<td></td>
</tr>
<tr>
<td>O14 PbHSP101</td>
<td>5' GGT CGG GGC AGA ATG TGC ACC ACT TCT ACT</td>
<td></td>
</tr>
<tr>
<td>O15 PbHSP101</td>
<td>5' GAT ATC GCC TCA TTA TTA TTT GAA TCA TCA</td>
<td></td>
</tr>
<tr>
<td>O16 PbHSP101</td>
<td>5' GCC GCC GGC CAC TTA TAG ATG ATG GGT CCT CTT G</td>
<td></td>
</tr>
<tr>
<td>O17 PbPTEX88</td>
<td>5' GGC AAG CTG CAT TCA GCA CAC ACT TTT TTA TAA AAT C</td>
<td></td>
</tr>
<tr>
<td>O18 PbPTEX88</td>
<td>5' CGG ATA CCG CTT TCT TTT TTT GAA TTT GTA C</td>
<td></td>
</tr>
<tr>
<td>O19 PbPTEX88</td>
<td>5' CCG ATG CGG GGC ACT GAG TTA CAG ATG AAT GTA</td>
<td></td>
</tr>
<tr>
<td>O20 PbPTEX88</td>
<td>5' CCG GAA TAC CAG AGG AGT GTC AAG TCG TAT TAA ATT C</td>
<td></td>
</tr>
<tr>
<td>O21 PbEXP2</td>
<td>5' TAG GCC GGC TTA GGA GCC TAA GGT TGT T</td>
<td></td>
</tr>
<tr>
<td>O22 PbEXP2</td>
<td>5' GTC GGG GTC TTA TAG GAA TAT GTC GGC CAA</td>
<td></td>
</tr>
<tr>
<td>O23 PbEXP2</td>
<td>5' CAT CGG GCC GGC ATG TTA AAG CAG CAC CTC</td>
<td></td>
</tr>
<tr>
<td>O24 PbEXP2</td>
<td>5' TAT GGC CCT TCC TAG TAA AAT TAC GAC GTC CCT ATC</td>
<td></td>
</tr>
<tr>
<td>O25 PbTRX2</td>
<td>5' GAT GCC CCT TCC TAG TAA AAT TAC GAC GTC CCT ATC</td>
<td></td>
</tr>
<tr>
<td>O26 PbTRX2</td>
<td>5' GGC ATA CCT TAA ATG GGT CAA GAC AAT GTA</td>
<td></td>
</tr>
<tr>
<td>O27 PbTRX2</td>
<td>5' CCC GGG GAA TAA GCT ATG CTA GAA GGT AT TAA ATT C</td>
<td></td>
</tr>
<tr>
<td>O28 PbTRX2</td>
<td>5' GCC GAA TAC CAG AGG AGT GTC AAG TCG TAT TAA ATT C</td>
<td></td>
</tr>
<tr>
<td>O29 PbEXP3</td>
<td>5' CCG CAG GGT GGA TCT CAC CAA GCC ATC</td>
<td></td>
</tr>
<tr>
<td>O30 PbEXP3</td>
<td>5' CAT GGC GGC CTA TTA TAG ATG ATG GGT CCT CTT G</td>
<td></td>
</tr>
<tr>
<td>O31 PbTRX2</td>
<td>5' TAG CCG CCT TCA GTC TAA AAA TCA CGG CTC TTA C</td>
<td></td>
</tr>
<tr>
<td>O32 PbTRX2</td>
<td>5' TAT GGG CCC CGG TAA TAT GTG CTT CAT GAT GA</td>
<td></td>
</tr>
<tr>
<td>O33 Pb101_5'R (Spe)</td>
<td>5' ACG CAC TAG TAC CAG GCA TAC GGT CTT TAA AAT C</td>
<td></td>
</tr>
<tr>
<td>O34 Pb101_3'F (Spe)</td>
<td>5' CCG GTA TGC CCT TGA CTA GTG GTG CAT ATG TAT CAT TGA</td>
<td></td>
</tr>
<tr>
<td>O35 Pb101_3'R (A)</td>
<td>5' GAC CCT AGG TCA CAA TGA AAG GGT TAT AAT AAC ATG</td>
<td></td>
</tr>
<tr>
<td>O36 PbTRX2 5_F(S)</td>
<td>5' GAG CGG CGG AAT AAC AAT TAG GTG GAC</td>
<td></td>
</tr>
<tr>
<td>O37 PbEXP2</td>
<td>5' CAT GCA TCA TCA TCA TCA GCA AAT C</td>
<td></td>
</tr>
<tr>
<td>O38 PbEXP2</td>
<td>5' TAT AAT AGA ATG TCA TCA TCA GCA A</td>
<td></td>
</tr>
<tr>
<td>O39 PbTRX2</td>
<td>5' TAT GGG CCC CGG TAA TAT GTG CTT CAT GAT GA</td>
<td></td>
</tr>
<tr>
<td>O40 Nanoluc</td>
<td>5' TAT CCT AGG ATG TGC TCC ACA TCA GCA</td>
<td></td>
</tr>
<tr>
<td>O41 Nanoluc</td>
<td>5' TGA CTC GAG TTA CGC CAG AAT GCG</td>
<td></td>
</tr>
<tr>
<td>O42 GFP</td>
<td>5' TGC GCT AGC ATG AGT AAA GGA GAA GAA CT</td>
<td></td>
</tr>
<tr>
<td>O43 EF1</td>
<td>5' CGG CAA ATG GAT CAC CTC TCT TTT TTT TTT TAA ATT C</td>
<td></td>
</tr>
<tr>
<td>O44 HA</td>
<td>5' CGG GCC GTG CAG CAC GCG TAC A</td>
<td></td>
</tr>
<tr>
<td>O45 PbPTEX150</td>
<td>5' GGA ATG GTT GAG GTT AAT GAG ACG G</td>
<td></td>
</tr>
<tr>
<td>O46 PbHSP101</td>
<td>5' GAG CGG CGG CAA AAT AAG ATA TTA TTA GGT TGA GAA</td>
<td></td>
</tr>
<tr>
<td>O47 PbPTEX88</td>
<td>5' GCC AAG CCT TAG AGC TCA ATA CAC ACA TCT GTG TAT TAA</td>
<td></td>
</tr>
<tr>
<td>O48 PbEXP2</td>
<td>5' TTA CTG CGG ATT CAC AAT TAG TGC</td>
<td></td>
</tr>
<tr>
<td>O49 PbEXP2</td>
<td>5' GAA AAC CCT AAA CAC ATT TGG TGC</td>
<td></td>
</tr>
<tr>
<td>O50 PbEXP2</td>
<td>5' TAT AAT AGA ATG TCA TCA TCA GCA A</td>
<td></td>
</tr>
<tr>
<td>O51 PbTR2</td>
<td>5' TGT TAG CTC ACC TGG TAA ATG ATG ATG ATG GTG C</td>
<td></td>
</tr>
<tr>
<td>O52 PbTR2</td>
<td>5' GCC AAG ATT GGA CTA GAC AAG CAG CAC GGA TTA TAT A</td>
<td></td>
</tr>
<tr>
<td>O53 PbTR2</td>
<td>5' GAC CCT AGG TAA ATG TCT CAA ATG GTC GTC TAA GTC</td>
<td></td>
</tr>
<tr>
<td>O54 PTEX88</td>
<td>5' GAG CGG CGG AAC AAT GAT CAA ATG TAT GAC CAA CAG ACG</td>
<td></td>
</tr>
<tr>
<td>O55 PTEX88</td>
<td>5' CAT GAC TAG TTT ACA TTA GGA GAT GTC GAA AAA TTA T</td>
<td></td>
</tr>
<tr>
<td>O56 PTEX88</td>
<td>5' TCC TAA TGT AAA CTA ATG GTG TTT TGG TCC AAA AG</td>
<td></td>
</tr>
<tr>
<td>O57 PTEX88</td>
<td>5' GAC CCT AGG ATG CTA AAT GTA CTA ATG CTT GTG C</td>
<td></td>
</tr>
<tr>
<td>O58 PdEXP2</td>
<td>5' CAC CCC GGC GGC GTG TAG CCT TAG TAA GTT GG</td>
<td></td>
</tr>
<tr>
<td>O59 PdEXP2</td>
<td>5' ATT TAC TAG GTG TGT ATG TTA GGT GCT CAC G</td>
<td></td>
</tr>
<tr>
<td>O60 PdEXP2</td>
<td>5' GCA TAC AAC ACT AGT AAA TAT TGG AAA ATG TGA CGC A TA</td>
<td></td>
</tr>
<tr>
<td>O61 PdEXP2</td>
<td>5' GAC CCT AGG AGC CTC ATT AGA ATG TCT TCT T</td>
<td></td>
</tr>
<tr>
<td>O62 PdTEX150</td>
<td>5' CAT GCG GCC CGG GTG GAA GTG GAA GTG GAA GTG G</td>
<td></td>
</tr>
<tr>
<td>O63 PdTEX150</td>
<td>5' CAT TAC TAG TGG CAT ATG GTG ATA AAT CAT AGA AGA</td>
<td></td>
</tr>
<tr>
<td>O64 PdTEX150</td>
<td>5' GAA GAT CTC GCA TTA ATG TCA CAA AGG GAT TCT CCT CCA</td>
<td></td>
</tr>
<tr>
<td>O65 PdTEX150</td>
<td>5' CGA CTG CAC TAG GTT CAT CCT CTT CAT CCT CGT CGT G</td>
<td></td>
</tr>
<tr>
<td>O66 PdTR2</td>
<td>5' ACT AGT CTC GAA TAA TAC TGT GGT TAG ATT A</td>
<td></td>
</tr>
<tr>
<td>O67 PdTR2</td>
<td>5' TTA CGG CAG CAG AGG ATG GTC AAC TG GGT TAG TAA G</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3

The conservation and characterisation of the *Plasmodium* Translocon of Exported Proteins
1.9 Introduction

Most of what is currently known about protein export in malaria parasites comes from work undertaken in *P. falciparum*. With respect to PTEX, its initial identification was described in *P. falciparum* and to date comprises of five protein components, three of which have been confirmed as genuine PTEX members (PTEX150, HSP101 and EXP2). Whilst PTEX88 and TRX2 co-affinity purified with these three components, PTEX88 and TRX2 still remain tentatively assigned as members of PTEX because validation that this interaction is genuine is missing.

Whilst the genes encoding the PTEX components are conserved across all *Plasmodium* species, providing some indication as to their importance, this does not necessarily imply the five orthologues interact to form a complex in other species as they do in *P. falciparum*. Importantly, whilst PTEX has been proposed as a protein translocation system, functional proof that it is directly responsible for parasite protein export is lacking.

This chapter aims to characterise the PTEX orthologues present in *P. berghei*, including whether PTEX88 and TRX2, along with PTEX150, HSP101 and EXP2 form a complex at the PVM. This species of *Plasmodium* was chosen as it supports the overarching aim of dissecting the role of PTEX in pathogenesis, which requires an *in vivo* model of malaria infection.
1.10 Results

1.10.1 PTEX components are expressed in *P. berghei*

PlasmoDB\(^{191}\) was used to identify the *P. berghei* orthologues of the *P. falciparum* PTEX components. Sequence alignments of each PTEX protein sequence from *P. falciparum* and *P. berghei* was then performed by Pairwise Sequence Alignment (EBI database), with between 46.2% to 90.6% amino acid identity observed between the *P. berghei* and *P. falciparum* PTEX protein sequences. HSP101 is the most highly conserved protein between the two species (Table 3.1). Similar to the *P. falciparum* PTEX components, all of the 5 PTEX orthologues in *P. berghei* have a signal sequence, which would be required for PV localisation. The putative *P. berghei* PTEX proteins are predicted to be a similar weight to the *P. falciparum* orthologue and the same features are observed in proteins from both species. For example HSP101 contains two ATPase domains, EXP2 contains conserved acidic repeats and TRX2 harbours a thioredoxin motif. Also, both *P. falciparum* and *P. berghei* PTEX88 and EXP2 have conserved cysteine residues (Figure 3.1). There is also evidence that the five PTEX genes are transcribed and expressed in *P. berghei* blood stages\(^{195}\) where protein export is known to occur.

In order to confirm these bio-informatic, transcriptomic and proteomic data, as well as to localise the putative *P. berghei* PTEX proteins within the parasite, several transgenic *P. berghei* parasite lines were generated in which each putative PTEX component was epitope tagged at the C-terminal end with a triple hemagglutinin (HA) and single Strep-Tactin II (Strep) epitope tag. Attempts to generate antibodies to several of the components were also undertaken.
1.10.2 Generation of reagents to \textit{P. berghei} PTEX components

Whilst rabbit polyclonal anti-PTEX antibodies have been generated against the \textit{P. falciparum} PTEX proteins, PfPTEX150, PfHSP101\textsuperscript{1} and PfEXP2\textsuperscript{174,196}, none had been developed for \textit{P. falciparum} PTEX88 and TRX2. Moreover, no antibodies existed against the \textit{P. berghei} PTEX orthologues. However, due to the regions where the \textit{P. falciparum} antibodies were targeted to, and the tight homology between the \textit{Plasmodium} PTEX orthologues shown in Figure 3.1, the \textit{P. falciparum} anti-PTEX antibodies have the potential to be cross reactive against the orthologous protein in \textit{P. berghei}.

Thus, the first step of this Chapter was to determine if the existing anti-PfPTEX antibodies were cross reactive against the putative \textit{P. berghei} PTEX components, before deducing if the generation of new anti-PbPTEX reagents was required. To achieve this, protein extracted from asynchronous PbANKA parasites was separated by SDS-PAGE, and probed in a Western blot with the available anti-PfHSP101, anti-PfEXP2 or anti-PfPTEX150 antibodies. As a comparative positive control for antibody reactivity, \textit{P. falciparum} 3D7 lysate was run alongside the PbANKA wild type lysates.

Figure 3.2 shows that the anti-PfHSP101 detects \textit{P. falciparum} HSP101 protein just under 100 kDa as expected, but also detects a \textit{P. berghei} protein orthologue running slightly higher, consistent with a predicted mass of the \textit{P. berghei} orthologue being 103.06kDa. Figure 3.2 also shows that the anti-PfEXP2 detects the \textasciitilde33 kDa EXP2 protein in \textit{P. falciparum} lysate, as well as a protein in \textit{P. berghei} above 25 kDa. This is smaller than the predicted molecular weight of the putative \textit{P. berghei} EXP2 protein of \textasciitilde31 kDa,
however, no other protein species were detected. Thus both the protein species detected by the anti-PfHSP101 and anti-EXP2 antibodies are likely to be the *P. berghei* orthologues of PfHSP101 and PfEXP2. Anti-PfPTEX150 did not recognise any protein species in the *P. berghei* lysate (Data not shown). Thus, taken all together, these experiments revealed that *P. falciparum* HSP101 and EXP2 antibodies could be used in *P. berghei* to detect the orthologous PTEX components by Western Blot (Figure 3.2). In contrast, anti-PfPTEX150 did not recognise any *P. berghei* protein species and antibody generation specific to the *P. berghei* orthologue was required. The reactivity of existing *P. falciparum* antibodies against the *P. berghei* orthologues is summarised in Table 3.2.

### 1.10.2.1 Attempts to generate antibody reagents against PTEX150 and TRX2

Given that the PfPTEX150 antibody was not cross-reactive against *P. berghei*, it was apparent that new *P. berghei* specific antibodies to PTEX150, as well as TRX2 and PTEX88, to which no antibodies are available for either species, were required. However, as part of this thesis, only PbPTEX150 and both PfTRX2 and PbTRX2 were targeted for antibody generation, as PTEX88 was being characterised in greater detail as part of another research project.

To express PbPTEX150 for antibody production, a region of the putative *P. berghei* PTEX150 gene constituting 470 base pairs of PbPTEX150 (Figure 3.1) was PCR amplified from PbANKA genomic DNA using the oligonucleotides O1/O2. The resulting fragment was digested with *Bam*HI and *Pst*I and cloned into the corresponding sites of the pB3 vector193 (Figure 3.3a). Likewise, to express PfTRX2 and PbTRX2 for antibody production, a region of
either TRX2 gene constituting 372bp of PfTRX2 and 315bp of PbTRX2 (Figure 3.1) was PCR amplified from either *P. falciparum* 3D7 or *P. berghei* ANKA genomic DNA, with the oligonucleotides O3/O4 or O5/O6 respectively, digested with *Bam*HI and *Pst*I and cloned into the corresponding sites of pB3 (Figure 3.3a). These regions of TRX2 lack the predicted signal peptide sequence but comprise the conserved thioredoxin domain (WCQAC). This cloning strategy incorporates a maltose binding protein (MBP) tag at the N-terminal end of PbPTEX150, PfTRX2 and PbTRX2 recombinant protein to maintain the solubility of the recombinant protein, and a 6-histidine (6xHis) tag at the C-terminal end to facilitate protein purification. The resulting plasmids were sequenced to ensure the coding region of PbPTEX150, PbTRX2, and PfTRX2 were error free and in-frame with MBP.

To express each PTEX protein, plasmids were independently transformed into *E. coli* BL21 bacterial cells, which were then grown to log phase and induced for protein expression with 1M Isopropyl B-D-1-thiogalactopyranoside (IPTG). The recombinant Pb-MBP-PTEX150-His and Pb-MBP-TRX2-His protein was then purified from the BL21 cells using an amylose resin and after a series of washes, the purified fusion proteins were eluted with 10 mM maltose, resulting in 9.5 mg of PfTRX2, 7 mg of PbTRX2 and 8 mg of PTEX150 protein. The integrity of the recombinant protein was subsequently assessed by SDS-PAGE and Coomassie staining. For the PbPTEX150 purification, the predominant protein species observed in the eluate was at about 63 kDa, which is consistent with the predicted molecular weight including the MBP-His tag (Figure 3.3b). For the PfTRX2 and PbTRX2 purification, the predominant protein species observed in the eluate was at about 59 kDa and 56, which was also consistent with the predicted molecular
weight including the MBP-His tag of 59.26 and 56.78 kDa respectively (Figure 3.3c, 3.3d). Unspecific protein species were not readily detectable in the elution fractions, suggesting the purification had been successful.

To generate polyclonal antibodies against PbPTEX150, PfTRX2 and PbTRX2, the recombinant protein was used to immunise rabbits and rats. Sera obtained from kill bleeds of animals that had received three immunisations unfortunately failed to detect a protein species in *P. berghei* or *P. falciparum* protein lysate at the expected molecular weight for TRX2 or PTEX150, and instead only recognised MBP (data not shown). With the PTEX150 recombinant protein, degradation was observed after short-term storage, and thus further attempts to generate antibodies against this protein were ceased. With the TRX2 recombinant protein, given that the MBP tag is significantly larger than TRX2 (43 kDa versus 14 kDa), it is likely that an immune response was predominantly induced towards the MBP portion of the recombinant protein rather than the target TRX2 protein.

Thus to ensure that only antibodies specific to either *P. falciparum* or *P. berghei* TRX2, and not to MBP were generated, 3 mg of the recombinant TRX2 protein was cleaved from the MBP tag with Factor Xa enzyme (FXa) prior to immunisation. As can be seen from Figure 3.4, full-length MBP-TRX2-6xHis protein runs at 43 kDa whilst cleaved TRX2-6xHis protein runs at approximately 14 kDa. However, un-cleaved full-length MBP-TRX2-6xHis protein was still present after an overnight incubation with FXa, indicating the enzyme was not efficient and did not completely cleave all the recombinant protein during this time. Therefore, in an attempt to purify TRX2 further, the gel fragment present at 14 kDa containing only the TRX2 protein was excised, solubilised and used to immunise a second batch of naïve rabbits.
Again, the sera from the second round of immunisations did not yield specific antibodies against either *P. falciparum* or *P. berghei* TRX2 protein as Western blot analysis of SDS-PAGE *P. falciparum* or *berghei* lysate failed to detect the expected protein species of 14 kDa. To determine if the sera contained even a low concentration of anti-TRX2 antibodies, IgG antibodies were purified and concentrated from the sera with Protein A - agarose beads. When this IgG purified antibody was probed against parasite protein lysate, it only resulted in lower detection of unspecific protein species and unfortunately TRX2 specific reactivity was not observed.

### 1.10.2.2 Peptide antibody development against TRX2

Considering previous attempts to generate antibodies against recombinant TRX2 protein failed, an alternative method to generate antibodies against TRX2 was performed. Here, commercial antibodies were raised by Abmart against short PfTRX2 peptides, selected for their epitope structure using Protein Surface Epitopes Targeted by Monoclonal Antibody Library (SEAL). The selection of nine independent epitopes was based on the likelihood that one might be reactive. Initially, *P. falciparum* TRX2 was chosen as the target species using this approach. If it proved to be successful, attempts using *P. berghei* specific epitopes would be considered since none of the peptides were predicted to be cross-reactive between *P. falciparum* and *P. berghei*. A total of nine peptide sequences were selected against PfTRX2 (Figure 3.5), and peptide monoclonal antibodies were generated (Abmart). Initially, two of the monoclonal antibodies detected a faint protein species at the expected size of TRX2 by Western blot analysis (data not shown), however, numerous attempts to repeat this observation failed. Further, IgG purification of the
antibodies against recombinant PfTRX2 protein did not enhance detection ability by Western blot analysis (data not shown).

1.10.2.3 Epitope tagging of PTEX components

Due to the unsuccessful attempts at generating polyclonal, monoclonal or peptide antibodies against the putative *P. berghei* PTEX proteins, an alternative method to characterise the PTEX components in *P. berghei* was explored. Accordingly, a series of transgenic *P. berghei* ANKA parasite lines were created in which the genes encoding the endogenous putative *P. berghei* ANKA PTEX components were tagged with a triple-HA and Streptactin II epitope tag, using a 3' replacement strategy. Even though the anti-PfHSP101 and anti-PfEXP2 cross-reacted against *P. berghei* lysate, the *P. berghei* EXP and HSP101 components were included in the epitope tagging process as a control for the antibodies, as well as to provide extra tools for analysis. This approach inserted the epitope tags at the C-terminal end of the protein without endogenous protein disruption (Figure 3.6a). To achieve this, approximately 2 kb of the 3' end of the target gene without the stop codon was cloned into the *SpeI* and *AvrII* of a modified epitope tagging construct termed pB3-150-HA/Str-DT3', upstream and in frame of the epitope tags. Flanking regions were designed to facilitate gene integration such that the full epitope-tagged PTEX gene would be expressed under its endogenous promoter.

Each of these five PTEX tagging constructs were sequenced to ensure the tag was in frame with the PTEX coding sequence (CDS). To facilitate integration, the plasmids were linearised within the target sequence at an engineered *SpeI* site, leaving ~1 kb of target sequence on either side of the
restriction cut site. Linearised DNA (1-2 µg) was transfected into schizont stage PbANKA parasites, which were then intravenously injected into naïve Balb/c mice. After mice were treated with pyrimethamine to select for transfectants, parasite growth was observed across all lines and at about seven days post infection when the parasitemia of infected mice reached approximately 5 % parasitemia, infected blood was harvested so that parasite genomic DNA and protein material could be isolated.

To determine whether correct gene targeting had occurred, diagnostic PCR was performed, using primers designed to distinguish between integrated and wild type parasites. The expected amplicon sizes representing 3xHA/Strep epitope-tagged PTEX genes were recovered (Figure 3.6b). To check for clonality, the presence of the corresponding wild type PTEX gene was assessed using the oligonucleotide combinations of O45 (150-HA), O46 (101-HA), O47 (88-HA), O48 (EXP2-HA) and O9 (TRX2-HA) with O44, which revealed that wild type parasites were present in each line. However, further cloning of parasites was not performed as transfected parasites could be discriminated by the presence of the HA tag. Subsequent confirmation of successful epitope tagging was obtained by Western blot analysis using mouse anti-HA antibodies against protein lysates made from transgenic parasites. Migration of the tagged PTEX protein species in all transfected parasite lines was in accordance with the predicted molecular mass and no labeling was observed in the un-transfected wild type control lysate (Figure 3.7a). Further to this, transgenic PbEXP2-HA, PbHSP101-HA parasites and wild type parasites were also probed with anti-PfHSP101 and anti-PfEXP2 antibody that had previously been shown in Figure 3.2 to cross-react to the P. berghei orthologous proteins. As expected, protein species that reacted with
the anti-PfHSP101 in the transgenic parasite lysates showed an increase in molecular weight in accordance with the addition of the 3xHA/Strep tag. However, for EXP2 the shift in molecular weight changed from 26 kDa in wild type parasites, to 35 kDa in EXP2-HA parasites, which is more than expected given the size of the epitope tags (~4.5 kDa), indicating EXP2 has an unusual mobility profile. Also, the reactivity of the -26 kDa species in PbEXP2-HA with anti-PfEXP2 indicates the presence of untagged EXP2 in the parasite population (Figure 3.7 b). In contrast, the PbHSP101-HA parasite line shows no visibly detectable untagged HSP101 protein and taken together with the PCR results that show a weak PCR result for the wild type locus, this indicates there are likely to be few wild type parasites in the population (Figure 3.7b). Importantly, the ability to successfully integrate the epitope tags into the loci of the PTEX genes indicates that all the PTEX genes in *P. berghei* are amenable to gene targeting.

### 1.10.3 *P. berghei* PTEX orthologues localise to the PVM

After generating the *P. berghei* PTEX HA-tagged parasite lines, the localisation of the PTEX components were analysed by immunofluorescence assay (IFA). This was performed on ring-stages parasites fixed with ice-cold acetone/methanol. For each parasite line, labeling with a rat anti-HA antibody and the anti-PfEXP2 antibody was performed (Figure 3.8). The PbEXP2-HA fixed parasites importantly showed that staining for HA and PbEXP2 co-localised, with a punctate labeling pattern observed outside the parasite periphery (Figure 3.8a top panel), similar to that observed with *P. falciparum* in which EXP2 localises to the PVM$^1$. This observation corroborates that the
PbEXP2-HA parasite line was appropriately HA epitope tagged, and that the anti-PfEXP2 antibody was indeed recognising endogenous EXP2 in *P. berghei*, despite the unexpected size of PbEXP2 observed in Western blots. In contrast, the anti-PfHSP101 antibody, which proved to be cross-reactive against *P. berghei* by Western blot analysis, did not clearly label Pb101-HA under IFA conditions (data not shown). Therefore, anti-PfHSP101 antibodies could not be used to colocalise HSP101 with other PTEX components on the PTEX-HA tagged parasite lines by IFA. As can be observed from Figure 3.8a, four of the PTEX proteins, PbPTEX150-HA, PbHSP101-HA, PbEXP2-HA, and PbPTEX88-HA, co-localised with PbEXP2. Therefore, it could be concluded that these proteins localise at the PV/PVM. This observation is consistent with previous localisations of PfPTEX150 and PfHSP101, but importantly, this is the first time PTEX88 has been localised in any *Plasmodium* species. The localization of TRX2 was less distinct and difficult to detect overall due to very weak labeling with the anti-HA antibody, perhaps due to the lower level of the expression of TRX2-HA protein. Whilst TRX2 did appear to be present with EXP2 in the early ring stages of the parasites blood stage, as the parasite matured into trophozoite stages, the localization of TRX2-HA appeared more internalized and towards the parasite periphery internal to the PVM. TRX2-HA localisation was also investigated in schizont stages generated from rings and trophozoite stages harvested from infected mice and further cultured *in vitro*. IFAs revealed TRX2-HA displayed punctate apical labeling in schizonts, reminiscent of the labeling pattern observed with other PTEX components in *P. falciparum*\(^1\) and PbPTEX150-HA included as a control (Figure 3.8b). Taken together, the IFAs confirmed that the PTEX orthologues in *P. berghei* are expressed and mostly co-localise at the PVM.
1.10.4 *P. berghei* TRX2 and PTEX88 interact with other PTEX components

Whilst co-localisation of the *P. berghei* PTEX orthologues at the PVM is consistent with being functionally equivalent to their *P. falciparum* orthologues, the IFAs performed above do not provide evidence that the components actually interact in *P. berghei*. Accordingly, co-immunoprecipitation experiments were performed to determine if the *P. berghei* PTEX orthologues interact with each other. Protein lysates from trophozoite or schizont stages expressing the *P. berghei* HA-tagged PTEX orthologues, or PbANKA wild type parasites, were affinity purified with rabbit anti-HA antibody coupled to magnetic beads and purified under native conditions. Post purification, the eluates were separated by SDS-PAGE and Western blots were probed with mouse anti-HA, anti-PfHSP101 or anti-PfEXP2 antibodies. As expected, the anti-HA antibodies did not detect any protein in wild-type lysate, but each of the five HA-tagged proteins of expected molecular masses were present in the elution fraction of the HA-epitope tagged transgenic parasites (Figure 3.9a).

In addition, affinity purification of PbPTEX150-HA, PbPTEX88-HA and PbTRX2-HA resulted in the co-precipitation of HSP101 (Figure 3.9b) and EXP2 (Figure 3.9c). Again, no signal was detected in the wild-type lysate eluates immunoprecipitated with anti-HA antibody. This indicates that the detection of PbHSP101 and PbEXP2 in the anti-HA eluates is specific. The observations that the PbPTEX150-HA and PbEXP2-HA immunoprecipitation co-precipitated with HSP101, and that PbPTEX150-HA and PbHSP101-HA co-precipitated with PbEXP2, is consistent with results in *P. falciparum* that show these proteins interact¹. Significantly, these experiments also revealed for the
first time, that that PbTRX2-HA and PbPTEX88-HA also specifically interact with HSP101 and EXP2 (Figure 3.9b and c).

A significant limitation with this analysis was the use of cross-reactive antibodies, originally designed for the *P. falciparum* PTEX orthologues. Whilst these antibodies are able to specifically and accurately detect *P. berghei* HSP101 and EXP2, the detection is significantly weaker than their *P. falciparum* counterparts, most likely due to the differences in the coding sequences between the species. Further to this, only HSP101 and EXP2 could be probed for due to the failure to generate specific reagents against PTEX150, PTEX88 and TRX2. Therefore, reciprocal immunoprecipitation experiment, such as an EXP2-HA pull-down followed with anti-PTEX88 detection, could not be performed to further validate the results.

Due to these limitations, further proteomic approaches were undertaken to confirm that TRX2 and PTEX88 interact with other PTEX proteins, as well as potentially finding other novel interacting partners of TRX2. Although both PTEX88-HA and TRX2-HA lines were available, only the TRX2-HA pull down was performed due to time restraints. Specifically, the elution fraction of the PbTRX2-HA or PbANKA wild-type parasites derived from anti-HA pull-downs, were separated by SDS-PAGE and resolved proteins were enhanced for visualization *in situ* with Sypro Ruby (Figure 3.10). Differences between protein species in the wild type and TRX2-HA protein fractions were observed. Corresponding gel fragments were excised from the wild type and PbTRX2-HA pull-downs in circumstances in which a protein species appeared in the PbTRX-HA fraction but not in the PbANKA fraction as the former were presumed to contain protein species that interacted specifically with TRX2-HA.
All samples were processed by capillary liquid chromatography coupled online with tandem mass spectrometry (LC-MS/MS) for peptide identification. Overall, gel slices excised from the TRX2-HA immunoprecipitation eluate yielded twenty-seven unique peptides to *P. berghei* PTEX orthologues, including PbHSP101, PbPTEX88 and PbEXP2, with 9% sequence coverage for each protein. In contrast, no PTEX peptides were detected in the corresponding *P. berghei* wild-type control (Figure 3.11a). These results further validate the immunoprecipitation experiments performed in *P. falciparum*. Interestingly, other proteins previously shown to affinity purify with *P. falciparum* PTEX components were also isolated (Figure 3.11b). These included the parasitophorous vacuolar protein 1 (PV1) and heat shock protein 70 (HSP70). Twelve additional proteins bound exclusively to TRX2-HA and were not present in the wild type eluate. Since their interaction is specific to TRX2, these proteins are potentially new or interacting components of PTEX. Non-PTEX peptides were recovered in matching bands of both the wild type and TRX2-HA eluates, consisting of mainly putative ribosomal proteins that are typically involved in the cellular process of translation, (eg: PbANKA_123250) and these represent proteins that have bound non-specifically to the anti-HA resin.
1.11 Discussion

The initial discovery of PTEX in *P. falciparum* was achieved using a range of proteomic, biochemical and cell biological approaches. In *P. falciparum*, PTEX comprises of PTEX150, HSP101 and EXP2, with TRX2 and PTEX88 remaining tentatively assigned constituents until their interactions with PTEX could be confirmed to be specific\(^1\). Further studies in *P. falciparum* have also validated the interactions between PTEX150, HSP101 and EXP2\(^{176}\).

Herein, database searches for the PTEX gene orthologues in other *Plasmodia* species revealed that the five PTEX genes are conserved across the genus, including *P. berghei*. Thus, if it could be confirmed that the putative PTEX components in *P. berghei* form a complex at the PVM in a similar fashion to that observed in *P. falciparum*, this would enable the functional dissection of PTEX in virulence and survival *in vivo* using *P. berghei* in a rodent model of infection.

Whilst specific antibodies have been generated against *P. falciparum* HSP101, EXP2 and PTEX150, only the anti-HSP101 and anti-EXP2 antibodies were found to be capable of recognising the *P. berghei* PTEX orthologues by Western blot to permit further characterization of these proteins in *P. berghei*. Of these two antibodies, only the one directed against EXP2 reacted against *P. berghei* in IFA. Thus several different attempts were made in this study to generate reagents that were capable of recognizing the remaining putative PTEX components in *P. berghei* by either Western blot analysis or IFA but unfortunately all attempts proved unsuccessful. It should be noted that although recombinant *P. falciparum* TRX2 has been successfully used by Sharma et al.\(^{186}\) to generate polyclonal antibodies, in our hands these
antibodies failed to react against native parasite protein by Western blot analysis. The difficulty in generating antibodies to TRX2 or the other PTEX components may be because the portion of the recombinant protein that was expressed is not very immunogenic. Alternatively, the native PTEX protein within the parasite may only be weakly expressed, rendering its detection difficult.

As a consequence of the failure to generate specific antibodies that could be used to characterize the putative PTEX components in *P. berghei*, all five PTEX proteins were instead epitope-tagged at their C-terminal end with a 3xHA/Strep tag, allowing the use of anti-HA antibodies for further studies. Importantly, the ability to tag each putative *P. berghei* PTEX component validated that the locus is amenable to gene targeting. IFA performed on each of the *P. berghei* transgenic PTEX-HA parasite lines from ring to late trophozoite stages revealed that PbPTEX150, PbHSP101 and PbPTEX88 co-localise with EXP2, the latter being the PTEX component most tightly associated with the PVM in *P. falciparum*. This indicates these components in *P. berghei* localise to where export machinery is expected to reside. However, the localisation of TRX2 during the ring stage could not be completely resolved, as in addition to co-localising with EXP2, PbTRX2-HA was also found within the parasite cytosol and potentially to an unknown structure. Nonetheless, PbTRX2-HA was also detected in schizonts, along with PbPTEX150, where it specifically located towards the apical end of each developed merozoite, consistent with TRX2 being carried in with the rest of the PTEX complex during parasite invasion\(^1\). Both the PVM and apical localisations observed here are in keeping with previous studies that show TRX2 is expressed in schizonts and present at either the PV/PVM during early
ring stages\textsuperscript{186,187}. Thus, whether the presence of TRX2 within the parasite infers a function in addition to its putative role in PTEX at the PVM remains unknown.

Using the newly generated HA-epitope tagged parasite lines as well as the anti-PfHSP101 and anti-PfEXP2 antibodies, it could be validated by immunoprecipitation experiments coupled with either immune-blot analysis or mass spectrometry that PTEX150, HSP101 and EXP2 interact to form a complex in \textit{P. berghei}. Importantly, this study was also able to confirm that PbPTEX88 and PbTRX2 are also part of the PTEX complex. Analysis of non-PTEX proteins that were identified as unique to the TRX2-HA pull-down and absent from the wild type negative control, revealed PbTRX2-HA also potentially interacts with the parasite vacuole protein 1 (PV1). This protein is unique to \textit{Plasmodium}\textsuperscript{198} and localises to the PV space\textsuperscript{197}, providing further support for the localization of TRX2 at the PV. \textit{P. falciparum} PV1 is amenable to gene tagging but attempts to disrupt the PfPV1 gene have been unsuccessful, indicating it is an essential protein\textsuperscript{197}. So far the function of PV1 is unknown, but its association with PTEX warrants further investigation as it may potentially be another \textit{bona fide} PTEX component.

Although the mass spectrometry analysis of proteins that affinity purified with TRX2-HA has shown interaction with other PTEX proteins and HSP70, a potential chaperone, it failed to yield any obvious novel proteins that may be the direct substrate of TRX2. Thus, without further experimentation on TRX2 (and indeed PTEX88 and PTEX150) its function within the PTEX complex still remains a mystery.
Table 3.1: Comparisons between the *P. falciparum* and the orthologous *P. berghei* PTEX protein

<table>
<thead>
<tr>
<th>PTEX Component</th>
<th>Gene I.D <em>P. falciparum</em></th>
<th>Gene I.D <em>P. berghei</em></th>
<th>Protein Identity (%)</th>
<th>Protein Similarity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEX150</td>
<td>Pf3D7_1436300</td>
<td>PbANKA_100850</td>
<td>30.1%</td>
<td>46.2%</td>
</tr>
<tr>
<td>HSP101</td>
<td>Pf3D7_1116800</td>
<td>PbANKA_093120</td>
<td>81.5%</td>
<td>90.6%</td>
</tr>
<tr>
<td>EXP2</td>
<td>Pf3D7_1471100</td>
<td>PbANKA_133430</td>
<td>61.0%</td>
<td>76.7%</td>
</tr>
<tr>
<td>PTEX88</td>
<td>Pf3D7_1105600</td>
<td>PbANKA_094130</td>
<td>44.0%</td>
<td>65.7%</td>
</tr>
<tr>
<td>TRX2</td>
<td>Pf3D7_1345100</td>
<td>PbANKA_135800</td>
<td>48.7%</td>
<td>75.9%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Protein similarities expressed as a percentage and generated by a Pairwise Sequence Alignment (Needleman-Wunsch alignment algorithm).
### Table 3.2: Reactivity of *P. falciparum* antibodies

<table>
<thead>
<tr>
<th>PTEX component</th>
<th>Reactivity of antibody against <em>P. falciparum</em></th>
<th>Reactivity of antibody against <em>P. berghei</em></th>
<th>Antibody generation attempted in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEX150</td>
<td>** IFA</td>
<td>- IFA</td>
<td>• No</td>
</tr>
<tr>
<td></td>
<td>*** Western analysis</td>
<td>** Western analysis</td>
<td></td>
</tr>
<tr>
<td>HSP101</td>
<td>** IFA</td>
<td>- IFA</td>
<td>• <em>P. berghei</em> PTEX150-polyclonal</td>
</tr>
<tr>
<td></td>
<td>*** Western analysis</td>
<td>* Western analysis</td>
<td></td>
</tr>
<tr>
<td>EXP2</td>
<td>*** IFA</td>
<td>*** IFA</td>
<td>• No</td>
</tr>
<tr>
<td></td>
<td>*** Western analysis</td>
<td>*** Western analysis</td>
<td></td>
</tr>
<tr>
<td>PTEX88</td>
<td>N/A</td>
<td>N/A</td>
<td>• No</td>
</tr>
<tr>
<td>TRX2</td>
<td>N/A</td>
<td>N/A</td>
<td>• <em>P. falciparum</em> TRX2 polyclonal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• <em>P. falciparum</em> TRX2 monoclonal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• <em>P. berghei</em> TRX2 polyclonal</td>
</tr>
</tbody>
</table>

*a* Comparative table showing antibodies against *P. falciparum* PTEX proteins and their cross-reactivity to the *P. berghei* orthologue. Symbols represent strength of antibody recognition (N/A, no antibody available; * no interaction, * weak interaction, ** medium interaction, *** strong interaction)
Figure 3.1: Schematic of the *P. falciparum* PTEX components and the orthologous proteins in *P. berghei*.

Colour codes correlate to distinguishing features and similarities between the two species. The protein length in amino acids (aa) is indicated. The regions to which antibodies were generated for PTEX150 and TRX2 are indicated by the green lines.
Figure 3.2: Anti-PfHSP101 and anti-PfEXP2 cross-react with P. berghei.

Western blot analysis of parasite proteins extracted from P. berghei ANKA and P. falciparum HSP101 parasites and separated by SDS-page using either (a) anti-PfHSP101 (1/250) or (b) anti-PfEXP2 (1/500) antibodies. The asterisks indicate P. berghei PTEX protein species where the antibody raised against P. falciparum is cross-reactive.
Figure 3.3: Expression of recombinant PTEX150 and TRX2 protein.

a) Schematic representation of the plasmid used to express PTEX150 and TRX2 in E. coli. MBP: maltose binding protein; 6xHIS: 6x histidine fusion protein. The green arrow indicates transcription start and red stop indicates transcriptional terminator. Jagged lines indicates the Factor Xa cleavage site IE/DGR.

b) Large-scale protein expression and purification of P. berghei PTEX150.

c) P. falciparum TRX2.

d) P. berghei TRX2 recombinant protein separated by SDS-page and stained with Coomassie. (-) Un-induced BL21 samples, show no production of fusion protein without the addition of IPTG, while as (+) samples induced with 1mM IPTG display efficient recombinant protein expression in BL21 E. coli cells. E1 and E2 indicate elution fractions of protein purified on nickel column. Arrowheads indicate expected molecular weight of the fusion proteins.
Figure 3.4: Cleavage of recombinant PTEX150 and TRX2 protein.  
**a)** Schematic representation of recombinant PTEX protein after incubation with Factor Xa resulting in the separation of the MBP fusion tag and the PTEX protein. MBP: maltose binding protein; 6xHIS: 6x histidine fusion protein. Broken jagged lines indicates the Factor Xa cleavage site  
**b)** Representative SDS-PAGE gel of post-cleaved recombinant TRX2 protein stained with Coomassie displays three protein populations; uncut MBP-TRX2-6xHIS at 57kDa, MBP at 43kDa and TRX2 at 14kDa, the latter of which was excised from the gel for rabbit immunisations.
Figure 3.5: Sequence alignment between *P. falciparum* and *P. berghei* TRX2.

Sequences in blue boxes represent the nine peptide *P. falciparum* sequences selected for monoclonal antibody generation. Peptides were selected using Monoclonal SEALTM Library Design Kit software and based on the most exposed regions of the sequence. Amino acid colour indicates residue classification. Degree of sequence conservation indicated by stars (identical residue; double dots (similar aa); single dot (weakly similar aa).
**Figure 3.6: Generation of *P. berghei* transgenic parasites expressing epitope tags.**

**a)** The targeting constructs were designed to integrate into the respective *P. berghei* PTEX locus by single-crossover recombination, such that the complete open reading frame was reconstituted while simultaneously introducing a combined C-terminal triple haemagglutinin (HA) and a single Strep-II (Str) epitope tag. The predicted structure of the endogenous locus before and after 3' integration is shown. Yellow box, 3xHA/Str epitope tags; SM, *Toxoplasma gondii* DHFR-TS selectable marker cassette; wavy lines, plasmid backbone; green arrow, transcription start site; stop, transcription terminator; SacII, linearization site.

**b)** Diagnostic PCR to detect integration into the PTEX locus. Oligonucleotide combinations O45-O49/O233R were used to detect incorporation of the epitope tags into transgenic PbPTEX150 (150-HA), PbHSP101 (101-HA), PbTEX88 (88-HA), PbEXP2 (EXP2-HA) and PbTRX2 (TRX2-HA).
Figure 3.7: Expression of *P. berghei* transgenic parasites with epitope tags.

a) Western blot analysis of parasite proteins extracted from WT and transgenic *P. berghei* parasites harvested from infected mice using anti-HA antibodies. 

b) Western blot analysis of WT and transgenic PbEXP2-HA and Pb101-HA using the indicated antibodies reveal that the anti-PfEXP2 and anti-Pf101 recognize a predominant protein species in WT *P. berghei* extracts at about the predicted molecular weight, while slightly higher species are observed in the transgenic parasite lines due to the incorporation of the epitope tags.
Figure 3.8: *P. berghei* PTEX components colocalise and have PV/PVM localization.
Double-labeling IFA performed on (a) fixed rings stages or (b) fixed schizont stages of *P. berghei* PTEX-HA transgenic parasites using the antibodies as indicated. BF: Bright field. ×1000 original magnification for all IFA images.
Figure 3.9: The five putative PTEX components form a complex in *P. berghei*, including PTEX88 and TRX2.

Immunoblot analysis of immunoprecipitations (IP) performed on *P. berghei* ANKA wild-type (WT) and transgenic HA-tagged parasite lysates using anti-HA antibody (α-HA) for the IP and probed with (a) anti-HA, (b) anti-Pf101 or (c) anti-PfEXP2. Supernatant (SN) was collected after parasite lysate was centrifuged and the flow fraction represents unbound supernatant material. The presence of PTEX components in the elution fraction of immunoprecipitations performed on HA-tagged parasites and their absence in the *P. berghei* wild-type (WT) control indicates the interaction is specific. Note that in the uncloned PbEXP2-HA transgenic population the EXP2 antibody detects both epitope-tagged and untagged EXP2 that associate in oligomers.
Figure 3.10: PbTRX2-HA interacts with unique protein species.
Elution fractions of *P. berghei* wild type (WT) and PbTRX2-HA parasite lysate pull downs with anti-HA, separated by SDS-page and stained with Sypro ruby. Unique protein species observed in the TRX2-HA fractions were excised along with the WT control and subjected to capillary liquid chromatography coupled online with tandem mass spectrometry (LC-MS/MS) for peptide identification.
Figure 3.11: PbTRX2-HA associates with PTEX & non-PTEX proteins.

a) Schematic representation of peptide sequence coverage of interacting PTEX partners of PbTRX2-HA represented as a percentage. No PTEX peptides were detected in the control WT fractions.

b) Representative graph of all unique peptides interacting with PbTRX2-HA that were not observed in WT. Included on the right-hand side are several peptides that were detected in both pull-downs and thus are likely to be interacting non-specifically with TRX2.
Chapter 4

Dissecting the role of the PTEX components and assessing parasite growth and virulence
1.12 Introduction

A study by Maier et al (2008) showed that approximately 25% of exported proteins could not be genetically targeted for disruption, indicating they are essential to the parasite. Thus any process which facilitates export of these proteins would also be expected to be essential.

Given the suspected role PTEX has in the protein export pathway, it is hypothesised that parasites lacking a functional PTEX would not survive. However, not all constituents of other translocons found in nature are essential for function and they can still translocate proteins even if some of the accessory components have been genetically disrupted. This chapter, therefore, aimed to genetically disrupt all five PTEX components to serve two purposes. Firstly, confirmation of the essentiality of PTEX to parasite survival would corroborate the essential role it is anticipated to have in protein export. Those PTEX genes that cannot be disrupted are therefore likely to make good anti-malaria drug targets. Secondly, if any PTEX components are amenable to gene deletion, this would indicate these components play an accessory role in PTEX function. Nonetheless, it would allow phenotyping of the resulting transgenic parasite lines to assess the consequences of altering PTEX function on parasite growth and virulence.

Attempts to genetically disrupt each PTEX gene was undertaken in *P. berghei*. Not only are transfections more efficient in this species compared to *P. falciparum*, if transgenic parasites could be derived, the mouse model for *P. berghei* allows assessment of PTEX’s role in parasite virulence and survival *in vivo*. In Chapter 3, it had been validated that PTEX is present as a complex in *P. berghei*, and importantly, that each PTEX gene is amenable to
targeting as they could be successfully HA-epitope tagged. In this Chapter, the construction of linear PTEX targeting vectors and their transfection into \textit{P. berghei} ANKA parasites is described, along with the analysis of recovered transgenic parasites in growth and virulence assays.

1.13 Results

1.13.1 Generation of PTEX knockout constructs

The PTEX targeting vector constructs were made using the pB3 plasmid\textsuperscript{193}, which comprises an ampicillin selectable marker to facilitate selection of plasmids in \textit{E. coli} and a \textit{Toxoplasma gondii} DHFR selectable marker (TgSM) cassette to select for transgenic parasites using the drug pyrimethamine. Appropriate restriction enzymes either side of the TgSM allowed for plasmid cloning (Figure 4.1). Final targeting constructs comprised the 5' and 3' UTR sequences of each respective \textit{P. berghei} PTEX gene to facilitate homologous recombination and replacement of the endogenous PTEX gene. The 5' and 3' PTEX targeting sequence flanks were obtained by PCR amplification of wild type \textit{P. berghei} ANKA genomic DNA using oligonucleotides O13/O14 and O15/O16 (HSP101), O21/O22 and O23/O24 (EXP2), O17/O18 and O19/O20 (PTEX88) and O25/O26 and O27/O28 (TRX2) (Table 2.1). The PTEX150 knockout construct was not created in this study as it has been previously described\textsuperscript{1}. The PCR products were digested with \textit{ApaI}/\textit{SacII} or \textit{BamHI}/\textit{XbaI}, respectively and cloned into the corresponding sites of the pB3 plasmid. The resulting constructs were transformed into \textit{E. coli} and transformants that grew under ampicillin selection were screened by PCR to assess if the ligation had been
successful. This was performed using oligonucleotides corresponding to either the 5' or 3' targeting sequence in conjunction with either oligonucleotide O30 or O29, respectively, which bind to the plasmid backbone. PCR positive clones were then subjected to further diagnostic restriction enzyme digest to confirm the 5' and 3' targeting sequence had been cloned (data not shown). Consequently, large-scale plasmid preparations were performed to provide enough DNA for *P. berghei* transfection.

To facilitate double-crossover homologous recombination into the *P. berghei* genome, plasmid DNA was linearised by digestion on either side of the targeting sequence with *Kpn*I and *Xba*I. Addition of *Pvu*I ensured the 2.87 kb backbone fragment was reduced to two smaller fragments to ensure no fully intact plasmids were present that could replicate as episomes within the parasite (Figure 4.1). Using the Amaxa transfection system, wild type schizonts were transfected with 2 μg of each linearised DNA fragment under the pre-set U33 program. Each transfection mix was administered intravenously into a single Balb/c mouse and 24 hrs post infection, mice were administered pyrimethamine drug water *ad libitum* to select for transgenic parasites.

### 1.13.2 Analysis of *P. berghei* transfectants

After positive selection with the antimalarial drug pyrimethamine, parasites transfected with a non-linearised PTEX knockout construct, which serves as a positive control, were visible in blood smears between days 5 to 7 post infection. However, after four weeks of drug selection, parasites transfected
with the HSP101 targeting construct could never be recovered and after two further unsuccessful transfection attempts, pursuits to generate a HSP101 knockout in *P. berghei* were relinquished. *P. berghei* parasites transfected with PTEX150, PTEX88, EXP2, and TRX2 knockout constructs all grew under pyrimethamine drug selection, however, the growth rate of these parasites were slow and occurred over a three week timeframe post transfection. At a parasitemia of 2-7%, infected blood was collected by cardiac bleed and parasite genomic DNA was extracted for analysis by PCR and Southern blot to determine if integration of the PTEX knockout construct into the PTEX locus had occurred (Figure 4.2a). Genotyping by diagnostic PCR for integration at the 5' and 3' ends revealed PTEX150, EXP2 and PTEX88 were refractory to gene deletion in three independent gene knockout attempts and parasites contained only episomal versions of the targeting construct (data not shown). These results were confirmed by Southern blot analysis of genomic DNA probed with either the 5' or 3' targeting regions (Figure 4.2b). Since the genes encoding HSP101, PTEX150, EXP2 and PTEX88 were all amenable to genetic targeting via 3' UTR replacement for the HA tagging strategy (Chapter 3, Figure 3.6), their refractoriness to gene deletion indicates that these four PTEX components are likely to be core translocon components whose expression is essential for parasite survival in the blood stages of malaria infection. Alternatively, these genes may provide a significant advantage in blood-stage growth, such that parasites containing a disruption in one of these PTEX genes became overgrown by wild type parasites possessing episomes.
1.13.3 The *P. berghei* TRX2 gene can be genetically deleted

Parasites transfected with the TRX2 knockout construct could be obtained on two separate occasions and were visible in Giemsa smears made at approximately 14 days post transfection. In contrast to the other four PTEX components, diagnostic PCRs on gDNA extracted from parasites transfected with the TRX2 knockout construct revealed correct integration had occurred on both occasions, with the presence of a 1.1kb (O9/O12) and 1.1kb (O10/O11) fragment indicative for 5’ and 3’ gene integration respectively (Figure 4.3). As expected, no DNA fragment using these oligonucleotide combinations was observed in wild type genomic DNA as only one primer in each pair is located in the endogenous locus. The resulting transgenic parasites were termed PbΔTRX2_1 and PbΔTRX2_2. To further confirm the integration of the TRX2 knockout plasmid, as well as if wild type parasites were still present, Southern blot analysis of PbΔTRX2_1 and PbΔTRX2_2 genomic DNA digested with *BglII* and *EcoRI* was performed, using Digoxigenin labeled probes generated against the 5’ and 3’ TRX2 flanking regions by PCR using oligonucleotides O25/O26 and O27/O28, respectively. The absence of a 4.9 kb species confirmed the loss of the wild type gene whilst the appearance of either a 5.8 kb (5’ targeting sequence as probe) or a 1.4 kb species (3’ targeting sequence as probe), respectively, corresponds to the expected gene knockout integration event (Figure 4.4a).

1.13.4 Creation of clonal *P. berghei* TRX2 gene knock out parasites

Prior to phenotypic analysis of the transgenic PbΔTRX2 lines, it was important to ensure that the transfectants were clonal and devoid of low
numbers of contaminating wild type parasites that could not be detected by the diagnostic PCR or Southern analysis. Accordingly, cloning of PbΔTRX2_1 and PbΔTRX2_2 was performed by intravenous injection of limiting dilutions of one to two parasites per each mouse. Of the six mice infected for each line, 83% and 50% of the mice became infected, respectively. To confirm which mice contained either wild type or knockout parasites, Southern blot analysis was performed on the isolated gDNA using the 5’ or 3’ PbTRX2 targeting sequence as probes (Figure 4.4b). All resulting clones harboured the disrupted TRX2 locus, indicating the TRX2 knockout parasites were clonal. Clone 1 of PbΔTRX2_1 and clone 3 of PbΔTRX2_2 were chosen for further analysis and will be referred to as PbΔTRX2_1.1 and PbΔTRX2_2.3 from here on.

1.13.5 Attempts to complement the *P. berghei* TRX2 knockout parasites

In order to validate that the loss of TRX2 protein was the cause of any phenotypic change that may be observed in the PbΔTRX2 knockout parasites in future experiments, an attempt to complement the TRX2 knockout was undertaken. For this, a gene replacement strategy was performed, whereby the TRX2 gene would be re-introduced into the endogenous TRX2 locus under the transcriptional control of its native promoter, and thus the genotype would be restored (Figure 4.5). To create the TRX2 knockout complement vector (PbΔTRX2c) a 3.0 kb fragment containing 1 kb of the *P. berghei* TRX2 5’ UTR, the complete TRX2 CDS and its 3’ UTR was obtained by PCR amplification of wild type *P. berghei*
genomic DNA using oligonucleotides O25/O28. The PCR product was digested with \textit{ApaI/EcoRI}, followed by the 3' overhang removal to allow blunt cloning into the pL0006 plasmid. Following ligation and transformation, positive clones were confirmed by diagnostic PCR and restriction enzyme digestion, after which two of the clones were sequenced to ensure the TRX2 CDS contained no mutations. The pL0006 plasmid comprises the human dihydrofolate reductive-thymidine synthase (hDHFR-TS) selectable marker gene cassette that encodes resistance to WR99210, which is administered via sub-cutaneous injection for three consecutives days post infection at 0.12 mg per injection. After four separate transfection attempts of the PbΔTRX2_1.1 with the complement plasmid, no parasites were recovered, regardless of whether the construct had been linearised to drive recombination into the PbΔTRX2 locus or not.

The lack of viable parasites in either the experimental (linearised plasmid) or control transfections (non-linearised plasmid), indicated that the PbΔTRX2 parasites were not coping with either the pressure of the second transfection or drug selection. Since a TRX2 complement parasite line was unable to be generated, subsequent phenotyping experiments were performed on both independent PbΔTRX2 knockouts simultaneously. Having two independent parasite lines behaving similarly maintains confidence that observations made in subsequent assays can most likely be attributed to the loss of the TRX2 protein, rather than as a result of a transfection anomaly, acquired mutations or other phenomenon.
1.13.6 The loss of TRX2 affects *P. berghei* growth *in vivo*

It was originally noted that when the PbΔTRX2 clonal lines were used to infect Balb/c mice for genotyping analysis, these parasites grew consistently slower than wild type parasites. Thus, to determine the rate of the growth defect observed in the PbΔTRX2 transgenic parasite lines, comparative growth experiments using Balb/c mice were performed. Twelve 6-week old naïve mice per group were infected with $1 \times 10^5$ *P. berghei* ANKA wild type, PbΔTRX2_1.1 or PbΔTRX2_2.3 parasites via intraperitoneal injection. Over the course of the experiment, the percentage parasitemia was determined by counting a minimum of 1000 erythrocytes in Giemsa-stained thin blood smears made on a daily basis. While parasites were visible by blood smears in all infected mice, already by day five the wild type cohort displayed a significant increase in parasitised erythrocytes (Figure 4.6). On the other hand, mice infected with either of the PbΔTRX2 clones displayed parasitemias around half that of wild type throughout the course of the infection. However, despite their delayed growth in mice, the PbΔTRX2 clones were ultimately able to generate high parasite loads. Beyond day 9-post infection, comparative analysis between groups could no longer be accurately performed as mice infected with wild type had to be humanely culled due to high parasite burdens. For instance, at day 11-post infection, the wild type parasitemia percentages averaged at 33.6% ± 4.3, with survival of the cohort only at 42%. This was compared to 100% survival for both the ΔPbTRX2 clones with parasitemia ranging between 21.3% ± 3.2 and 24.2% ± 2.4. Indeed, comparative infection experiments of the parasite lines in Balb/C mice confirmed that loss of TRX2 had a significant impact on growth.
To further examine the PbΔTRX2 growth delay and pinpoint where the defect was likely occurring, synchronous in vivo infection assays were performed. For this, new invasion events were monitored over three cycles of infection; beyond this it is unreliable as the infection becomes naturally asynchronous. Accordingly, wild type and PbΔTRX2_1.1 infected blood containing mostly ring-stage parasites from Balb/c mice infected between 5-10% parasitemia was cultured in complete RPMI media for 16 hrs. Cultures were then harvested and purified schizonts were mechanically ruptured to release merozoites. The homogenous mix was then filtered twice through a 0.22 μm syringe filter to purify the individual merozoites. Merozoite preparations, devoid of contaminating schizonts were added to freshly extracted naive mouse erythrocytes and invasion was allowed to proceed for 30 min at 37 °C under vigorous shaking. Equal numbers of erythrocytes infected with wild type or PbΔTRX2 parasite lines were then intravenously administered into individual Balb/c mice and blood smears were analysed every 2 - 4 hrs for the next three cell cycles.

While newly invaded ring-stage parasites were observed 23 hrs after infection with wild type parasites, PbΔTRX2_1.1 took an extra 5 hrs to reinvade erythrocytes and to start the second blood cycle (Figure 4.7). A similar delay was observed during the second cycle, such that the PbΔTRX2_1.1 now lagged about 8 hrs behind the wild type parasites. Furthermore, despite similar numbers of wild type or PbΔTRX2 infected erythrocytes being administered to mice, the parasitemia of PbΔTRX2_1.1 were less than half that of wild type parasites during the second cycle, indicating that either parasites were dying or being cleared by the immune system.
### 1.13.7 Assessment of PbΔTRX2 growth *in vitro*

As trophozoite and schizont stages sequester in the host microvasculature in the *in vivo* experiments described above, analysis of these parasite stages is not possible. Therefore, to look at growth of all parasite stages, an *in vitro* invasion assay was repeated, but on this occasion, purified merozoites that had newly invaded erythrocytes were kept in culture in complete media, rather than injected into mice, until mature schizonts formed. At every two hrs following invasion (T=0), thin smears of the cultures were made. The close sampling of the time points ensured the morphology of the developing parasites could be monitored and enabled examination of the stage of the cycle at which the loss of TRX2 has its greatest impact (Figure 4.8a). Within each time point, parasites were counted and scored according to their development and morphology as indicated in Figure 4.8b.

A total of one hundred parasitised erythrocytes were counted, with results represented as the percentage of different stages in the population over time. The most obvious delays occurred at the beginning and end of the cell cycle (Figure 4.8c). At 4.5 hrs post invasion, only 2.9 % of wild type parasites remained at early ring-stage, whereas 75 % of PbΔTRX2_1 and 30 % of PbΔTRX2_2 were still at this stage. By 7.5 hrs post infection, the majority of wild type had progressed into early trophozoite stage (73 %), with no early rings remaining whereas 33 % and 13 % of PbΔTRX2_1 and PbΔTRX2_2, respectively, had still not moved beyond the early ring stage. Likewise, by 24 hrs post infection, when 83 % of the wild type parasites had already entered the schizont stage (43 % early and 40 % late schizonts), only 15 % of PbΔTRX2_1.1 and 20% of PbΔTRX2_2.3 were at a similar stage of parasite development, with low numbers of late schizonts formed, if any. However,
examination of the mature schizonts used to prepare the merozoites for the
*in vitro* invasion assay did not reveal a difference in the number of
merozoites per schizont between the wild type and PbΔTRX2_1.1 (Figure
4.8d).

### 1.13.8 Loss of TRX2 leads to alterations in parasite virulence

Since PTEX is implicated in the export of virulence proteins and PbΔTRX2
clones are shown to consistently cause reduced infection rates, a correlation
between disruption of PTEX and reduced parasite virulence was explored.
To assess this, C57/Bl6 mice were used for infection experiments comparing
wild type and PbΔTRX2 knockout lines, each infected with $1 \times 10^6$ parasites
via intraperitoneal injection. C57/Bl6 mice were chosen because when
infected with *P. berghei* ANKA they succumb to a severe lethal form of
malaria, termed experimental cerebral malaria or ECM. In humans cerebral
malaria is associated with a multitude of neurological symptoms and
extensive underlying pathology. Typically, cerebral malaria symptoms
observed in mice and scored in this study include staring of fur, ataxia,
hunching and paralysis, with symptoms typically occurring between days 6-
10 post infection. Upon displaying the symptoms described, mice are
humanely culled as the cerebral malaria onset is irreversible and death
occurs within hours. Mice that do not succumb to cerebral malaria by this
time become affected by anemia due to high parasite loads.

In this study, the C57/Bl6 mice infected with wild type parasites rapidly
succumbed to cerebral malaria symptoms around 7 days post infection,
whereas mice infected with the PbΔTRX2 clones showed a delay in
developing ECM, if at all (Figure 4.9a). For example, by day 7 post infection, 83% of mice from the wild type group had to be culled because of the ECM symptoms they displayed. In comparison, mice infected with either the PbΔTRX2_1.1 and PbΔTRX2_2.3 at day 14 had only a 33 % and 66 % death rate, respectively. It was at this point that the experiment was ceased, however, as it was difficult to establish whether the death of PbΔTRX2 infected mice deaths was due to cerebral malaria or in fact anemia (Figure 4.9b). A log-rank (Mantel-Cox) test was used to calculate statistical significance suggesting that comparisons between the both the wild type and PbΔTRX2_1.1 and wild type and PbΔTRX2_2.3 survival curves were significant (P<0.001).

The parasitemia of the infected C57/Bl6 mice were also examined and the ΔPbTRX2 clones showed delays in parasitemia similar to what was observed in Balb/c mice (Figure 4.6 and 4.7). While wild type mice exhibited a mean parasitemia of 9.1% ± 0.9 on day 6-post infection (1 day prior to developing ECM), by day 9 post infection when all but one wild type mouse had been culled, mice infected with PbΔTRX2_1 exhibited a mean parasitemia of only 5.0% ± 0.5 and mice that survived infection with PbΔTRX2_2 had a mean parasitemia of 5.6% ± 1.6 (Figure 4.9b). Moreover, even when the PbΔTRX2 clones reached higher parasitemia, this did not influence their ability to cause ECM. Together the comparative infection experiments of the parasite lines in C57/Bl6 mice confirmed that the loss of TRX2 had a significant impact on parasite virulence.
1.14 Discussion

Whilst the PTEX machinery is a promising anti-malaria drug target, it is important to validate which components of PTEX are essential for parasite virulence and survival as these would serve the best targets for future drug development. In order to achieve this, attempted gene knockouts of all five PTEX genes in \textit{P. berghei} were undertaken via a homologous recombination strategy. This study revealed that \textit{P. berghei} HSP101, EXP2, PTEX150 and PTEX88 could not be genetically disrupted after multiple attempts and thus they are likely to all be essential or are at least required to significantly enhance parasite growth and survival. In contrast, Matz et al (2013)\textsuperscript{199} were able to derive transgenic \textit{P. berghei} lacking PTEX88 and although these parasites were viable, they exhibited significant growth defects. In their case, the PTEX88 knockout parasites harboured a fluorescence cassette, enabling them to be positively selected using flow cytometry, which was not the approach taken here.

Based on the prediction that HSP101 provides the crucial energy source for protein unfolding prior to translocation through the putative pore-forming component, EXP2, it was not unexpected to find that HSP101 and EXP2 are essential \textit{Plasmodium} proteins and indeed a recent study has shown that inducible knockdown of HSP101 in \textit{P. berghei} is lethal to the parasite\textsuperscript{200}. Likewise, PTEX150 has not been able to be genetically deleted in \textit{P. falciparum}\textsuperscript{7} and inducible knockdown of PTEX150 in \textit{P. falciparum} is lethal, thus the inability to knockout PTEX150 in \textit{P. berghei} is consistent with these results. However, the lack of orthologues and distinctive protein domains for PTEX150 as well as for PTEX88 outside the \textit{Plasmodium} genus precludes assignment of their function at this stage. Nonetheless, based on the make-
up of other translocon systems in prokaryotes and eukaryotes, which usually comprise chaperones, receptors and structural components, in addition to the pore-forming component and the molecular motor\textsuperscript{189}, we envisage that PTEX150 and PTEX88 most likely serve as structural components of PTEX or potentially act as a receptor for cargo to dock prior to translocation across the PVM.

In contrast, the fifth PTEX component, TRX2, was able to be genetically disrupted with successful parasite recovery, and thus \textit{P. berghei} TRX2 is a non-essential PTEX component. However, the marked reduction in growth and virulence phenotypes of parasites lacking TRX2 confirms this protein plays an important blood-stage role and suggests TRX2 plays a key regulatory role in PTEX function. Although the two Pb\textit{Δ}TRX2 clones varied slightly in their potential to cause ECM as well as in their growth rates, it is important to note that both independent Pb\textit{Δ}TRX2 clones consistently grew significantly slower and displayed altered virulence when compared with the wild type line on all occasions. The reasons for the difference between the lines is unclear but may be related to the number of times the clones had been passaged. Additionally, Pb\textit{Δ}TRX2 parasites displayed consistent growth delays \textit{in vivo}, potentially as a result of infected erythrocytes being cleared more efficiently by the spleen, particularly if TRX2 contributes to the export of virulence proteins that are involved in sequestration. Importantly, the reduced growth rates \textit{in vivo} as measured from blood parasitemia, do not appear to be the result of fewer merozoites produced per schizont, indicating the TRX2 disruption does not affect the parasites ability to reach schizont stages, but rather, Pb\textit{Δ}TRX2 parasites took longer to progress through the cell cycle (as indicated by both \textit{in vitro} and \textit{in vivo} experiments) and fewer
PbΔTRX2 parasites also made it through each cycle in vivo. Whether this is a result of some parasites dying each cycle due to poorer nutrient uptake or increased clearance of infected RBC by the spleen for example awaits further investigation. Interestingly, the most obvious growth delays of the PbΔTRX2 parasites occurred during schizont and ring stages, which correlates with when TRX2 expression is initiated and when PTEX is predicted to be functional, respectively. Although repeated attempts to genetically complement PbΔTRX2 parasites by using a second selectable marker were unsuccessful (probably due to the initial poor health and growth of the PbΔTRX2 parasites), it should be noted that both independent PbΔTRX2 clones consistently demonstrated similar phenotypes in each assay.

TRX2 is a small ~13 kDa protein that belongs to the thioredoxin family of proteins that acts to reduce disulphide bonds in substrate proteins via a conserved CXXC active site. Recombinant P. falciparum TRX2 has been shown to reduce insulin in vitro, indicating that it has redox activity. Interestingly, both chloroplasts and mitochondria use redox signals to regulate protein translocation across their respective membranes. For example, in chloroplasts, redox signals modulate the formation and reduction of inter- and intra-molecular disulphide bonds of translocon components at the outer membrane to regulate protein import. Additionally, the redox state of the stroma is sensed by the translocon at the inner chloroplast membrane and when there is a high demand for redox-related proteins, the efficiency of translocation of this subset of proteins is adjusted accordingly. As EXP2 and PTEX88 contain numerous conserved cysteine residues (Figure 3.1),
PTEX could also be regulated in a redox fashion to adjust protein export into the host cell cytosol, with TRX2, the most obvious candidate to perform this function. Since some of the exported proteins are predicted to be involved in nutrient uptake, this could account for the PbΔTRX2 clones exhibiting reduced growth rates. Alternatively, TRX2 may control the formation of disulphide bonds in a particular subset of exported cargo and thus regulate the ability of these proteins to be exported. Although exported cargo containing disulphide bonds in *P. berghei* have not been characterised, in *P. falciparum*, such cargo could include PfEMP1 and STEVORS, which play a role in parasite virulence. Future studies will require identification of the TRX2 substrate proteins to elucidate which of these alternate functions TRX2 may have in the PTEX complex or whether TRX2 performs another function altogether.
Figure 4.1: Construction of pB3-PTEX knockout constructs.
Schematic of the original pB3 plasmid for constructing the knockout plasmids. The plasmid contains a *Toxoplasma gondii* DHFR selectable marker cassette (TgSM) and an ampicillin selectable marker (AMP). The 5' and 3' untranslated regions (UTRs) specific to each of the five PTEX genes were PCR amplified and cloned into the appropriate restriction enzyme sites. The final targeting construct was linearised at *KpnI*, *XbaI* and *PvuI* restriction enzyme sites prior to transfection.
Figure 4.2: HSP101, PTEX150, EXP2 and PTEX88 are not amenable to gene deletion.  

**a)** Schematic of the *P. berghei* targeting constructs designed to integrate into the respective endogenous PTEX locus by double crossover recombination. The predicted structure of the endogenous locus before and after integration is shown. TgSM indicates *Toxoplasma gondii* DHFR selectable marker cassette;  

**b)** Southern blot analysis of genomic DNA extracted from *P. berghei* wild type (WT) and parasites surviving pyrimethamine selection. Digestion with *Bgl*II or *Bgl*II/*Eco*RI and hybridized with either probes made from the 5' or 3' targeting sequence reveal that the endogenous gene (E) remained intact and bands indicative of an integration event (In) were not observed. Molecular weights are indicated.
Figure 4.3: Genetic disruption of TRX2 P. berghei.

a) Schematic of the P. berghei endogenous locus, the final P. berghei TRX2 knockout targeting construct and the P. berghei TRX2 locus after integration. Black arrows indicate oligonucleotides used for diagnostic PCRs. Restriction enzyme sites BglII (B) and EcoRI (E) and expected Southern DNA fragment sizes are indicated.

b) Diagnostic PCR for 5’ & 3’ integration of genomic DNA extracted from pyrimethamine resistant parasites.
**Figure 4.4:** Confirmation of the *P. berghei* TRX2 gene knockout

**a**) Southern blot analysis of genomic DNA extracted from *P. berghei* wild type (WT) and TRX2 knockout transfectants (PbΔTRX2_1 and PbΔTRX2_2) digested with *BglII* and *EcoRI* and hybridised with either the 5’ (left panel) or 3’ (right panel) targeting sequence as a probe. Molecular weights are indicated, with (E) indicating endogenous and (IN) indicating integration locus.

**b**) Southern blot analysis of *P. berghei* wild type (WT) or PbΔTRX2 positive clones digested with *EcoRI/BglII* and probed with either the 5’ (left panel) or 3’ (right panel) targeting sequence as a probe.
Figure 4.5: Construction of a *P. berghei* TRX2 complement plasmid.
Schematic of the *P. berghei* endogenous locus, the final *P. berghei* TRX2 knock-in targeting construct (PbΔTRX2KOc) and the expected *P. berghei* TRX2 locus after integration.
Figure 4.6: Loss of TRX2 in *P. berghei* leads to reduced parasite loads *in vivo*.
Comparative growth analysis of *P. berghei* wild type and both PbΔTRX2 clones in infected Balb/C mice (n=12). The cross indicates number of deaths. Error bars represent SEM and statistical significance was calculated using a two-tailed t-test. *P<0.05, **P<0.01.
Figure 4.7: Loss of TRX2 in *P. berghei* results in a longer parasite cycle *in vivo*.

*In vivo* growth analysis of PbΔTRX2 parasite line in which invasion had been synchronized *in vitro* prior to intravenous inoculation in mice. The solid red and blue dashed arrows indicate the time points at which new invasion events of wild type (WT) and PbΔTRX2_1, respectively, could be detected.
Figure 4.8: Loss of TRX2 leads to altered growth phenotypes in vitro.

a) Giemsa-stained blood smears taken throughout the asexual blood cycle. *In vitro* growth analysis over 24 hrs of *P. berghei* parasite lines following synchronous invasion *in vitro*. The first invasion events were observed at 0.5 hrs post invasion (p.i.).

b) Representative morphology of parasites that were used for scoring the stage of growth.

b) Representative panel of either wild type or PbΔTRX2_1 infected erythrocytes seen in Giemsa smears from invasion to schizont development *in vitro*. The stages are expressed as a ratio observed in 100 infected erythrocytes counted.

d) Column graph depicting the number of merozoites formed per mature schizont (n = 50).
Figure 4.9: Loss of TRX2 in *P. berghei* leads to altered virulence and growth phenotypes in C57/Bl6 mice. 

a) Growth curves and b) survival curves (n = 14) of *P. berghei* ANKA, PbTRX2_1.1 and PbΔTRX2_2.3 (n = 12) in C57/Bl6 mice. Data were pooled from two separate experiments. Error bars represent SEM. A two-tailed t-test was used to calculate statistical significance between wild type and PbΔTRX2 clones. ***P<0.001.
Chapter 5

Functional evidence that PTEX is involved in parasite protein export
1.15 Introduction

Chapters three and four of this thesis have demonstrated that the five-membered PTEX complex is present at the PVM in *P. berghei* and confirmed its importance to parasite survival and/or growth and virulence through attempts to delete the respective PTEX genes. Yet despite the strong circumstantial evidence that PTEX is the protein export machinery, direct proof for a functional role of PTEX in parasite protein export is absent.

This Chapter aims to characterise PTEX function and its relationship with protein export. It will closely investigate the difference in the protein export levels between wild type parasites and a transgenic parasite line that has a disruption in the accessory PTEX component, TRX2.

To achieve these aims, the development and optimisation of methods for assessing parasite protein export in *P. berghei* is required, as only a limited number of antibodies are available to exported proteins in this species. These assays are necessary to directly quantitate individual exported proteins as well as protein export at a more global level.

1.16 Results

1.16.1 The deletion of *P. berghei* TRX2 deletion does not affect the export of the endogenous PEXEL proteins PBANKA_122900 & PBANKA_114540

To determine whether disruption of TRX2 affects parasite protein export, specific antibodies against known endogenous exported proteins,
PbANKA_114540 and PbANKA_122900 were used against wild type and PbΔTRX2 parasites in immunofluorescence analysis. Both of these proteins contain the PEXEL motif (RxLxE/Q/D) and localise to punctate structures in the erythrocyte cytosol\(^9\)
 Figure 5.1a). Following incubation of wild type parasites fixed with 4 %PFA/0.01 % glutaraldehyde in rabbit sera containing antibodies to either PbANKA_114540 or PbANKA_122900, and subsequent labeling with anti-rabbit Alexa Fluor 488 antibodies, punctate labeling was observed within the infected erythrocyte cytosol (Figure 5.1b). A similar labeling pattern was observed when PbΔTRX2 parasites were analysed by IFA, although there was a slight fluorescence reduction in the host cell cytosol of PbΔTRX2 infected erythrocytes (Figure 5.1b). However, quantitation of the cell fluorescence levels in both the erythrocyte and parasite cytosol by calculating the corrected total cell fluorescence (CTCF) using ImageJ revealed that there was no significant difference in the amount of labeling with anti-PbANKA_122900 (Figure 5.1c) or anti-PbANKA_114540 (Figure 5.1d) between wild type and PbΔTRX2 infected cells. Moreover, there was no accumulation of either protein at the PV or PVM, indicating these two proteins could be successfully exported beyond the confines of the PVM and into the cytosol of erythrocytes infected with PbΔTRX2.

1.16.2  \textit{P. berghei} TRX2 knockout parasite lines expressing an episomally-expressed exported reporter protein

To develop alternative ways of assessing protein export in the PbΔTRX2 parasite line, a reporter construct was generated and introduced into wild type and PbΔTRX2 parasites. The reporter construct encodes an exported
chimeric GFP. This reporter construct comprises the N-terminal coding sequence of KAHRP containing its signal sequence plus PEXEL motif (RxLxQ) fused in frame to GFP, with expression driven under the *P. berghei* EF1α promoter and the transcriptional terminator derived from the *P. berghei* Calmodulin gene. This reporter has been previously shown to be exported in PbANKA wild type parasites\(^95\). The construct also contains the human DHFR cassette to enable selection of parasites transfected with this construct using the drug WR9910. This reporter construct is termed p35.EF1α.K+GFP.CAM.

After transfection of PbANKA wild type and PbΔTRX2 with p35.EF1.α.K+GFP.CAM and drug selection on day 1 and 3 post infection, after drug treatment, wild type parasites containing the GFP expression construct were already visible in peripheral blood smears at day 8 post infection. However, PbΔTRX2 parasites were not visible until day 16, by which time the transfected wild type lines had already been culled as the mice exhibited high parasitemias.

So that the transgenic parasite lines could be imaged for GFP export at the same time to ensure consistency between the analysis, wild type and PbΔTRX2 WR9910-resistant parasites containing the episome after the secondary transfection were instead infected from liquid nitrogen stocks into naïve Balb/C mice. At six days post infection, inspection of fluorescence in wild type parasites showed they expressed and exported GFP into the erythrocyte cytosol (data not shown). In contrast, the levels of GFP expression in the cytosol of PbΔTRX2 infected erythrocyte was clearly reduced, although it was also noted that GFP expression could not be detected in the parasite cytosol either (data not shown). This lack of fluorescence indicated that GFP might be poorly expressed by the PbΔTRX2
parasites rather than the parasite having a reduced capacity to export proteins. This observation could be attributed to the PbΔTRX2 parasites growing slowly and fluorescence measured after drug administration when parasitemias are low, and thus the nearly undetectable fluorescence in PbΔTRX2 was most likely due to the episome being lost through parasite replication. Due to the limitations of this reporter assay, further approaches were sought to determine if there were changes in export capacity between wild type and PbΔTRX2 parasites.

1.16.3 **Generation of a *P. berghei* TRX2 knockout parasite line containing an integrated exported GFP reporter gene.**

After multiple attempts to transfect the PbΔTRX2 parasite line with an episomal exported GFP reporter failed, an attempt was made to regenerate the PbTRX2 knockout parasites again, however, this time integrating an exported GFP reporter construct simultaneously. This would eliminate the need for a second transfection and secondary drug selection.

To generate the TRX2 knockout/exported GFP reporter construct, pFRT-2A-mCherry/KAHRPL-GFP (derived from p3'regFRT\(^{203}\)) was modified to include regions of homology specific to the *P. berghei* TRX2 gene sequence (Figure 5.2a). The wild type exported GFP-expressing construct contained the PbTRX2 5' UTR region, which was amplified from *P. berghei* using oligonucleotides O31/O32 and incorporated into the vector at SacII and Apal sites. This construct already contained the GFP coding sequence in frame with the N-terminal sequence of KAHRP, including its signal sequence and PEXEL motif under the regulatory control of the *P. berghei* HSP70 promoter.
Downstream of GFP, the *P. berghei* TRX2 3' UTR, which had been amplified by PCR with oligonucleotides O66/O67, was cloned into the *Mlu*I and *Sac*II restriction enzyme sites (Figure 5.2a). The construct was linearised with *Sac*II such that the TRX2 5' and 3' UTRs would facilitate double homologous integration into the PbTRX2 locus when transfected into PbANKA wild type parasites, leading to disruption of TRX2 locus and insertion of the GFP reporter. In addition, a second construct was created in which the entire PbTRX2 CDS under its endogenous 5' UTR promoter was PCR amplified, using oligonucleotides O31/O39 and cloned into the *Sac*II and *Not*I site of p006/FRT (Figure 5.2b). Downstream of this, the *P. berghei* thrombospondin-related anonymous protein (TRAP) (PBANKA_134980) 3' UTR was inserted. Integration of this construct into the *P. berghei* TRX2 endogenous locus would result in the replacement of TRX2 with a fully functional TRX2 gene and integration of the exported GFP reporter, to serve as a positive control for imaging analysis.

PbANKA wild type parasites transfected with either reporter construct could be recovered after pyrimethamine selection. Parasites containing the TRX2 replacement/reporter construct were visible in peripheral blood smears at day six post infection, while the parasites harbouring the TRX2 knockout/reporter construct were visible at day 10. These parasite lines are subsequently referred to as WT+GFP and PbΔTRX2+GFP, respectively. Diagnostic PCRs on gDNA extracted from WT+GFP and PbΔTRX2+GFP using oligonucleotide O9/O43 and O42/O10, revealed integration had occurred in both lines, with the presence of a 3.4 kb or 1.5 kb and 1.6 kb species indicative for 5' and 3' gene integration respectively (data not shown). As expected, no DNA fragment was amplified in the wild type
fractions with these oligonucleotide pairs, however, primers specific for wild type gDNA (O39/O10) amplified the expected 1.16 kb fragment in the wild type control (data not shown).

Southern blot analysis of WT+GFP and PbΔTRX2+GFP genomic DNA digested with EcoRI/BglII or NdeI/BglII was performed, where Digoxigenin labeled probes were generated against the 5' TRX2 flanking regions using oligonucleotides O5/O6. In the EcoRI/BglII digestion, the absence of a 4.8 kb species representing the wild type locus and appearance of either a 5.8 kb for WT+GFP or a 3.9 kb for PbΔTRX2+GFP species was observed. In the NdeI/BglII digestion, the absence of a 5.3 kb species representing wild type and appearance of either a 7.2 kb for WT+GFP or a 3.9 kb for PbΔTRX2+GFP species was observed (Figure 5.2c). These bands correspond to the expected sizes for gene replacement or gene knockout event, respectively.

1.16.4 Live-cell fluorescent imaging of wild type or TRX2 knockout parasites expressing an exported GFP reporter

As PbΔTRX2+GFP parasites still exhibited a growth delay compared to WT+GFP, a synchronous time course assay was performed with WT+GFP and PbΔTRX2+GFP to analyse GFP expression. For this, two naïve mice were infected with liquid nitrogen stocks of WT+GFP and PbΔTRX2+GFP and administered pyrimethamine drug treatment for five days. Both mice were then cardiac bled and infected blood was cultured until parasites reached schizont stage. To ensure synchronicity, purified schizonts were mechanically ruptured to release merozoites by gently passing the
homogenous cells through a 0.22 µm filter twice. Merozoites were then allowed to invade naïve mouse erythrocytes by vigorous shaking at 37 °C. Cells were washed in media, and centrifuged gently to pellet the erythrocytes and to remove un-invaded merozoites. Invasion of naïve red cells was efficient for both wild type and knockout parasites, with counts of invasion events revealing that the rate of invasion was comparable between the two lines (data not shown).

Infected erythrocytes were then put back into in vitro culture at 37 °C and sampling and live cell imaging was performed every 6 hrs (Figure 5.3). At the first time point of 8 hrs post invasion, wild type parasites were exporting GFP into the host cell and by 11 hrs, when parasites were at trophozoite stage, intense GFP fluorescence was observed in the erythrocyte cytosol. Contrary to this observation, PbΔTRX2 parasites appeared to have limited GFP export in the first 8 hr time point, with the majority of GFP still present at the parasite vacuole. However, upon further parasite development, knockout parasites appeared to export the GFP in a similar fashion as wild type parasites, such that by 16 hrs post invasion, when parasites were at mid to late trophozoites, GFP was exported beyond the PVM and into the host red cell cytosol. Together this suggested that in the initial stages of infection, PbΔTRX2+GFP parasites showed slower protein export, but by the time the parasites made it to trophozoite stage, they could export GFP just as efficiently as wild type parasites.
1.16.5 Generation of parasites harboring an exported Nanoluc reporter construct

Unlike with GFP, the detection of the protein Nanoluc is exquisitely sensitive, thereby permitting very low levels of Nanoluc protein to be measured. This opened up the possibility to transiently transfect wild type and PbΔTRX2 parasites with an exported Nanoluc reporter, which would enable protein export to be compared between the two lines within 1-2 cycles. Two versions of the Nanoluc constructs were designed; pEF1α.PfKAHRP+.NL.CAM, which is expected to be exported due to the presence of the KAHRP signal sequence and PEXEL motif (Figure 5.4a), and pEF1α.NL.CAM, where Nanoluc is retained within the parasite (Figure 5.4b). In both constructs, Nanoluc expression is driven under the PbEF1α promoter and the 3’ UTR is that from the P. berghei Calmodulin gene. Nanoluc was PCR amplified from pNL1.1[Nluc] (Promega) using oligonucleotide O40/O41. The 534 base pair Nanoluc amplicon product was cloned into the existing p35.EF1.α.K+GFP.CAM in vector pl0035204 plasmid at the AvrII and XhoI restriction enzyme sites replacing the GFP.

To test if the PbΔTRX2 parasite line was defective in exporting Nanoluc protein, both wild type and PbΔTRX2 parasites were cultured and grown for transfection procedure as previously described. Each parasite line was split before transfection, so that half the prepared parasites were transfected with either circular pEF1α.K+.NL.CAM or pEF1α.NL.CAM. Consequently, four new parasite lines were generated; wild type with exported Nanoluc (WT+NL), wild type with non-exported Nanoluc (WT-NL), PbΔTRX2 with exported Nanoluc (PbΔTRX2+NL) and PbΔTRX2 with non-exported Nanoluc (PbΔTRX2-NL).
As a positive control, wild type parasites were also stably transfected with the Nanoluc constructs and treated with pyrimethamine rather than WR99210 post-transfection. This line was used for differential lyses optimization (described below) to test the fluorescence intensity of the Nanoluc protein. Wild type parasites containing either episome (PbWT±NL_PYR) grew normally compared to wild type parasites without an introduced construct. From preliminary analysis, high levels of Nanoluc activity in excess of $1 \times 10^6$ RLU was measured in 2 μl of blood infected with wild type parasites that were stably expressing either the exported or non-exported Nanoluc.

To maintain the plasmids within the parasite after transfection, WR9910 was administered by sub-cutaneous injection at days 1 and 3 post transfection. Whilst this approach was taken with the episomal GFP construct, as previously described, the parasitemia levels do not need to be high to detect Nanoluc. Even though the PbΔTRX2±NL parasite lines grew slower than the PbWT±NL parasites, it did not affect the measurement of Nanoluc protein, as results were expressed as a ratio between the PbΔTRX2+NL and the PbΔTRX2-NL control. At days 2-4 post-transfection, parasites were harvested from mice by tail bleeds (5 μl), allowing multiple sampling at different time points.

To differentiate between Nanoluc in the parasitophorous vacuole or host cell fraction, also referred to as non-exported or exported Nanoluc, respectively, a pore-forming toxin, equinatoxin (EQII) was used to compromise the red cell membrane without destroying the parasite membranes. This process releases the soluble host cell cytosolic fraction of
exported Nanoluc without impacting the parasite vacuolar membrane, thereby maintaining non-exported Nanoluc within the parasitophorous vacuole. To release the Nanoluc within the parasite, the pellet fraction was washed and then completely lysed by hypotonic lysis. Equal amounts from each fraction and from each parasite line were taken and combined with NanoGlo for quantification of illumination.

Tail blood from both WT+NL and PbΔTRX2+NL at comparable parasitemias of 0.5-1% were sampled when parasite development was between mid-rings to late trophozoite stages, followed by measurement of Nanoluc activity using a luminometer (Bertholdt). To calculate the export percentages, the relative light units (RLU) determined from the host cell fraction was divided by the total RLU obtained from the parasite cytosol fraction. Data from three separate experiments, where asynchronous parasites were sampled, was collated and is shown in Figure 5.4c, whereby the wild type and PbΔTRX2 export percentages show that no significant difference (p=0.0897) was observed between the two parasite lines.

1.16.6 Measurement of global protein export in *P. berghei* using semi-immune sera

So far, the assays described above measure the export of single parasite proteins or reporter genes. As PbΔTRX2 parasites are viable, it is likely the parasites are still able to export proteins, therefore the ability to distinguish export at the individual protein levels may be difficult. However, the cumulative export response may be expected to be much greater and thus to assess this, a global protein export assay was undertaken. This involved
quantifying proteins exported to the infected erythrocyte surface using semi-immune sera made specifically against *P. berghei* infected erythrocytes, in which antibodies are made to parasite derived antigens presented on the erythrocyte surface.

To test the reactivity of the newly generated semi-immune sera, PFA fixed wild type and PbΔTRX2 infected erythrocytes were assessed for labeling with either non-immune (negative control) or semi-immune sera (Figure 5.5a). Wild type infected red cells do not exhibit surface staining when exposed to sera from naïve mice that have never been exposed to a malaria infection. However, when the same wild type infected sample was exposed to semi-immune sera (Figure 5.5b), intense labeling was present at the erythrocyte cell periphery, indicative of recognition of parasite surface antigens. Whilst the PbΔTRX2 infected erythrocytes still reacted to the semi-immune sera, the level of labeling observed was greatly reduced (Figure 5.5c).

To quantify the level and differences of surface labeling of erythrocytes infected with wild type versus PbΔTRX2 using semi-immune sera, flow cytometry was used. For FACS analysis, wild type and PbΔTRX2 infected erythrocytes at similar parasitemias ranging from 2-4% were obtained from the tail vein of infected Balb/c mice. Samples were collected from asynchronous infections, when parasites were between mid-rings to mid-trophozoite stage. Triplicate blood samples were incubated with either *P. berghei* semi-immune or non-immune sera diluted 1:20 in blocking solution (1% casein/RPMI). After three washes with block solution, cells were incubated for one hr with goat anti-mouse IgG AlexaFluor 647 diluted in block solution. Samples were then washed a further three times followed with a
brief incubation in Sybr safe nucleotide stain. Cell preparations were analysed with a FACS Canto II (BD Biosciences). Quantitative FACS analysis of erythrocytes harvested from asynchronously infected mice show that the two independent clonal populations of PbΔTRX2 knockout parasites exhibit significantly reduced levels of surface labeling with *P. berghei* semi-immune sera compared with wild type parasites. Over six assays, (with each parasite line prepared in triplicate), wild type export was normalised to a hundred percent export ability, and export in knockout parasites was expressed relative to this. PbΔTRX2_1 and PbΔTRX2_2 displayed a reduction in export ability of about 50% and 25% respectively (Figure 5.6).

Whilst a comparable parasitemia between the different sample sets were used for the above analysis, there was the potential for bias given that the PbΔTRX2 grows slower and thus less mature stages that potentially have exported less were examined in the PbΔTRX2 lines. Therefore to ensure that FACS analysis was performed on the most comparable parasite stages, a synchronous time-course assay was performed. For this, synchronous mouse infections were initiated by injecting a high number of filter-purified merozoites into the tail vein of mice. At the second infection cycle, the parasitemia across the two infections were about 1-2% for wild type and PbΔTRX2 and still synchronous. Blood samples for FACS analysis were taken from the same infection cycle, but repeated at six-hr intervals between the wild type and PbΔTRX2_1, so that export of wild type and the slower growing PbΔTRX2 could be analysed at similar stages of growth.
To monitor the parasite growth and development between the lines, Giemsa smears were taken at the time FACS analysis was performed (Figure 5.7a). This revealed that at the same time point, the development of PbΔTRX2 was less advanced than the wild type and more comparable to the wild type time point taken 6 hrs earlier. Therefore the level of export observed at 15 hrs post infection for the PbΔTRX2 was compared to that of wild type measured at 8 hrs post infection. Even when this was taken into consideration, the surface labeling of the PbΔTRX2 parasites was significantly reduced when compared to wild type parasites in independent experiments by about 50% (n=6) (Figure 5.7b).

1.16.7 Structural and morphological changes to *P. berghei* infected erythrocytes

Host cell remodeling by *P. falciparum* also involves structural and morphological changes to the erythrocyte, such as the appearance of knobs on the surface of *P. falciparum* infected red cells. Considering that a *P. falciparum* orthologue of KAHRP is not expressed in *P. berghei*, it is not known whether *P. berghei* infected erythrocytes show obvious morphological changes to the erythrocyte by scanning electron microscopy. However since *P. berghei* infected cells can evade the immune system by sequestering, it is possible that changes to the *P. berghei* infected erythrocyte surface do occur and hence altering PTEX function may lead to loss of this phenotype, which could be explored by comparing wild type and PbΔTRX2 strains.

The difficulty with assessing modifications to an infected erythrocyte is the need to ensure only infected cells are isolated and imaged. Hence, for this
analysis the infected erythrocyte population needed to be enriched to yield >90% purity. To achieved this, infected erythrocytes were cultured in vitro until the majority were mid to late trophozoite. Erythrocytes harboring trophozoite stage parasites were specifically isolated using a MACS magnetic column, while uninfected erythrocytes and ring stage parasites were eluted. As can be seen in Figure 5.8a, uninfected erythrocytes appear smooth when imaged by SEM. However, when wild type infected cells were compared to uninfected erythrocytes there were morphological differences. Generally, infected red cells were rigid, lumpy and contained surface protrusions. Whilst observations here showed fewer protrusions than has been previously observed in P. falciparum, the surface of PbANKA infected erythrocytes were clearly different to that of uninfected cells. Thus, this method was then used to look at the overall effect of deletion of TRX2 on the morphology of erythrocytes. The PbΔTRX2 parasitised cells appeared to be most comparable to uninfected red cells, with a reduced degree of surface protrusions and cell rigidity when compared to wild type. However, on occasion, PbΔTRX2 parasitised cells did appear to contain randomised protrusions. This effect could be attributed to the reduction of overall protein export or even a subset of specific proteins responsible for this protrusion effect that are not being exported in the PbΔTRX2 parasite line. One hundred random erythrocytes from each parasite line were quantitated for their surface protrusion, resulting in wild type infected cells containing approximately 85% more protrusions than observed on PbΔTRX2 infected cells, which when analysed by unpaired t-test was significant (P<0.0001)(Figure 5.8b).
1.17 Discussion

The third aim of this research project involved addressing the functional contribution of PTEX to protein export. Whilst *P. berghei* provides many benefits in studying the malaria pathogen *in vivo*, phenotypic analysis of a *P. berghei* PTEX knockout is difficult without a large bank of antibody reagents, including to exported proteins. Attempts to phenotypically compare wild type and *PbΔTRX2* parasites were problematic since assays to measure changes in protein export in *P. berghei* were not developed and thus novel reagents and methodologies first had to be established.

Unfortunately, the ability to examine the export phenotypes of the *PbΔTRX2* clones by transfection of chimeric exported (versus non-exported) GFP reporter constructs were unsuccessful as the *PbΔTRX2* parasites, which grow slower than the wild type, struggled to survive and maintain episomes. To circumvent this, the TRX2 knockout was re-derived such that a GFP reporter cassette was integrated into its genome. Whilst expression of exported GFP was apparent in both the wild type and *PbΔTRX2* parasites, a delay in GFP expression and export into the host erythrocyte was observed in the parasite line lacking TRX2. The delay in GFP expression relayed to about 4-6 hours, which was in keeping with the growth delay of the *PbΔTRX2* parasites described in Chapter 4. Thus, whether the reduced GFP expression in the *PbΔTRX2* parasites is the result of the parasites growing more slowly and having less time to export proteins or whether the slower growth stems from a reduction in protein export is difficult to discern. Ultimately, by trophozoite stages, the *PbΔTRX2* parasites were able to export similar levels of GFP as wild type parasites. Interestingly, no GFP
accumulation was observed within the PV, which would be expected if there were a block in the export process.

Only recently have *P. berghei* PEXEL-containing proteins been identified\textsuperscript{109,147} and demonstrated to be exported by IFA or by live cell imaging. In this study two antibodies that recognize the endogenous exported proteins, PbANKA\textsubscript{114540} and PbANKA\textsubscript{122900}, which both harbour PEXEL motifs, were used to study protein export (C. K. Moreira, B. Naissant, A. Coppi, L. Bennet, E. Aime, B. Franke-Fayard, C. J. Janse, I. Coppens, P. Sinnis and T. J. Templeton, personal communication). While both anti-PbANKA\textsubscript{114540} and anti-PbANKA\textsubscript{122900} recognised parasite exported proteins in the host erythrocyte, there did not appear to be any significant affect on export of these proteins in PbΔTRX2 infected cells.

All together, observations made with the endogenous antibodies and both GFP and Nanoluc reporter constructs indicated that there was no overall significant change in the export of the individual proteins. However, since the PbΔTRX2 parasites are viable, it is not surprising that parasites would still be able to export proteins but export efficiency could be affected. However, even a slight decrease in export potential or delay in export may be difficult to identify at the individual protein level. Compounding this is that as the role of TRX2 in the complex is still unknown and thus it is highly possible that only a particular subset of protein cargo would be affected by a TRX2 deletion or that TRX2 only plays a more significant role under particular environmental conditions that parasite is faced with (for example, febrile episodes when the parasite undergoes heat shock). Thus, without reagents to a wide variety of different exported proteins, these experiments became rather limited.
With this in mind, further investigation into export deficiency in the PbΔTRX2 parasite line led to the development of semi-immune sera as this would provide a means to measure the global set of exported parasite proteins exposed on the erythrocyte surface. Overall results using the semi-immune sera by IFA and FACS analysis clearly demonstrated that there was a reduction in global protein export in the PbΔTRX2 parasites. To further corroborate that the changes observed were due to export deficiency rather than a slower growing PbΔTRX2, the synchronous time course assay was developed whereby export levels were analysed when parasites were at comparable stages, ensuring no bias was given to PbΔTRX2 parasites that were not at their maximum export potential. Even when taking the growth delay into account, the PbΔTRX2 parasites displayed a reduced export phenotype of about 50% when compared to wild type parasites. These findings are in keeping with the suspected role of TRX2 as an accessory PTEX member. Since parasites lacking TRX2 are still viable, they have the potential to export proteins, but whether they are capable of exporting all cargo albeit at reduced levels or whether only a particular non-essential subset of proteins is blocked from being exported awaits further investigation.

Along with establishing the loss of parasite protein export in a line with a defect in PTEX, determining whether this affect in protein export results in morphological changes to the infected erythrocyte was explored. Whilst normal erythrocyte morphology is affected in *P. falciparum* infections, no such changes have been described for *P. berghei*. Much of this is due to the dogma that KAHRP is responsible for the erythrocyte protrusions, and without KAHRP present in the *P. berghei* genome, no other known proteins
have been considered to alter the morphology of \textit{P. berghei}-infected cells. Whilst the specific cause and mechanisms are unknown in \textit{P. berghei}, infected erythrocytes have been shown to sequester\textsuperscript{147}. With this in mind, it is not unreasonable that \textit{P. berghei} also expresses cytoadhesive ligands on the erythrocyte surface that enables the parasite to sequester and that this also alters the morphology of the infected erythrocyte. Interestingly, preliminary scanning electron microscopy showed that \textit{P. berghei} wild type infected cells, exhibited surface protrusions and a deformed morphology whereas the PbΔTRX2 infected erythrocytes were morphologically more similar to uninfected cells.

Taken together, this work demonstrates that blocking optimal PTEX function through the genetic deletion of TRX2 leads to a reduction in global parasite protein export and if this includes a reduction in the expression of cytoadhesive ligands on the infected erythrocyte surface, this may also explain why fewer C57/Bl6 mice infected with PbΔTRX2 succumbed to cerebral malaria than mice infected with wild type \textit{P. berghei}. It may also explain the reduced growth rates \textit{in vivo} as the parasites may have been more rapidly cleared from the circulation. It would therefore be interesting to look at the spleens of infected mice and sections of tissue harvested from organs such as the brain, lung and adipose tissue, to investigate the capacity of the PbΔTRX2 parasites to sequester \textit{in vivo}. 
Figure 5.1: *P. berghei* TRX2 deletion does not affect the ability to export two identified endogenously expressed exported PEXEL proteins.

a) Schematic representation of the localisation of PBANKA_122900 & PBANKA_114540 in a *P. berghei*-infected erythrocyte. b) Representative IFA of intraerythrocytic parasites, showing no obvious blockage of export of PEXEL proteins PBANKA_122900 & PBANKA_114540 in PbΔTRX2 infected cells compared to wild type. c & d) Quantitation of fluorescence intensity (CTCF) of images presented in (b) shows no significant differences between PbΔTRX2KO compared with wild type infected erythrocytes (n=100). Error bars represent SEM for all replicates.
Figure 5.2: Generation of a *P. berghei* TRX2 knockout encompassing a GFP reporter.

a) Schematic of the targeting constructs designed to integrate an exported GFP reporter into the endogenous *P. berghei* TRX2 locus by double crossover recombination. The predicted structure of the endogenous locus before and after integration is shown. b) Schematic representation of the complemented gene replacement. Integration of either construct into wild type parasites results in GFP that should be exported based on the KAHRP signal sequence and PEXEL motif (KsP). hDHFR indicates human DHFR selectable marker cassette. c) Southern blot analysis of genomic DNA extracted from *P. berghei* WT, WT+GFP and PbΔTRX2+GFP parasites after pyrimethamine selection. Digestion with EcoRI/BglII or Ndel/BglII and hybridised with the 5’ targeting sequence probe show that the endogenous gene (E) is present in wild type parasites but is missing in both transgenic parasites, and bands indicative of an integration event (I) were observed at the predicted sizes in WT+GFP and PbΔTRX2+GFP. Molecular weights are indicated.
Figure 5.3: Time-course of GFP expression and export in transgenic parasites. Representative images of live cell GFP expression of either wild type or PbΔTRX2 infected cells, taken at sequential time-points after parasite invasion. In PbΔTRX2, an initial delay of GFP export is observed at 8hrs post infection, however by 16hrs post infection, GFP export in the *P. berghei* TRX2 knockout parasites is equivalent to that of wild type. BF, Bright field.
Figure 5.4: Schematic of the constructs designed for episomal expression of Nanoluc in *P. berghei*. 

a) Exported Nanoluc (with PEXEL and signal sequence) and b) Non-exported Nanoluc (no PEXEL or signal sequence). hDhFR SM indicates human DHFR selectable marker cassette; Green arrows indicates transcription start and red stop indicates the transcription terminator. KsP indicates KAHRP signal sequence & PEXEL motif. c) Nanoluc (NL) export calculated as a percentage of NL in erythrocyte cytosol divided by the total NL expressed. Exported NL measured as a percentage between wild type and PbΔTRX2 (n=16) parasites harboring NL expression plasmid showing no significant difference in export capacity.
Figure 5.5: Generation of semi-immune sera against *P. berghei* infection. Representative IFA of wild type infected erythrocyte surface expressed antigens reacted with a) non-immune or b) semi-immune sera. c) The PbΔTRX2 infected cell surface is partially recognised by the semi-immune sera. The original magnification for all IFA images was ×1000.
Figure 5.6: Disruption of *P. berghei* TRX2 leads to reduced protein export on the erythrocyte surface.
Ratios of the erythrocyte surface labeling was determined from asynchronous infections by flow cytometry using non-immune sera staining as a baseline (for background labeling) and with wild type export percentages set at 100%. Quantitative FACS data shown graphically of wild type (n=7) and PbΔTRX2_1 (n=7) and PbΔTRX2_2 (n=6) whereby parasites with the TRX2 gene deletion exhibit significantly reduced levels of surface labeling with *P. berghei* semi-immune sera compared with wild type parasites (*P=0.05; **P=0.01; ***P=0.001, unpaired t-test).
Figure 5.7: Disruption of *P. berghei* TRX2 leads to reduced protein export in synchronous infections.

a) Giemsa smears showing the stages of parasite development at time points relative to when wild type parasites had invaded erythrocytes. PbΔTRX2 parasites exhibit significantly reduced levels of surface labeling with *P. berghei* semi-immune sera compared with wild-type parasites. b) Quantitative FACS analysis of erythrocytes harvested from synchronous mouse infections initiated by injection of purified merozoites into the tail veins of mice. Surface labeling of infected erythrocytes with semi-immune sera was performed at time points relative to when the wild-type parasite line reinvaded erythrocytes for the second cycle post invasion. Taking into consideration that disruption of TRX2 leads to slower growth by about 6 hrs, the surface labeling of PbΔTRX2 parasites at a stage of growth comparable to that of wild type parasites is also significantly reduced (n=3 independent experiments). (*P=0.05; **P=0.01; ***P=0.001, unpaired t-test).
Figure 5.8: Surface modification of infected erythrocytes

a) Representative images after scanning electron microscopy of uninfected, PbANKA wild type or PbΔTRX2 infected erythrocytes. Differences observed between the uninfected and wild type infected cell surface in the form of knob-like protrusions extending from the erythrocyte membrane. PbΔTRX2 infected cells resemble the uninfected control.

b) No of protrusions counted on the surface or either wild type or PbΔTRX2 infected erythrocytes observed under SEM, showing a significant reduction in protrusions on the erythrocyte surface of PbΔTRX2 infected cells. Data pooled from multiple samples prepared on different days (n=100).
Chapter 6

Discussion and future directions
Using a number of different approaches in this study, it could be confirmed that orthologues of the *P. falciparum* PTEX orthologues exist in the rodent malaria species, *P. berghei*. Additionally, it provided validation that that PTEX88 and TRX2 are *bona fide* constituents of PTEX. This confirmation provided a solid grounding for further analysis of *P. berghei* PTEX components in vivo, to assess their role in both parasite virulence and survival and to confirm whether PTEX is indeed responsible for exporting parasite proteins.

Despite multiple attempts to genetically disrupt *P. berghei* EXP2, HSP101, PTEX150 and PTEX88, all were unsuccessful. In contrast, TRX2 could be genetically deleted on numerous occasions. These findings are consistent with those of Matz et al.\(^{199}\), with the exception that they were also able to knockout *P. berghei* PTEX88 in addition to TRX2, most likely because they employed much more sensitive parasite recovery methods that involved the selection of fluorescent knockout parasites by FACS sorting. Given that EXP2, HSP101 and PTEX150 could be epitope-tagged, the inability to knockout these genes suggests that they are essential to the parasite. Formal proof of this has come from a recent paper that used gene regulatable systems developed for *P. falciparum* and *P. berghei* to knockdown expression of PTEX150 and HSP101, respectively.\(^{200}\) In the case of PTEX150, a transgenic parasite was derived in which the PTEX150 locus was modified to incorporate a glucosamine-inducible glmS ribozyme within its 3' UTR. Upon addition of glucosamine to the culture media, cleavage of the 3' UTR by the ribozyme hastened degradation of PTEX150 mRNA, leading to parasite death. In the case of HSP101, transgenic parasites were derived in which the HSP101 coding sequence was placed under the transcriptional
control of an anhydrotetracycline (ATc)-regulated transactivator element\textsuperscript{205}, and thus HSP101 mRNA levels could be regulated by the addition of ATc to the drinking water of mice. Similar to the PTEX150 knockdown, reduction of HSP101 transcript levels was lethal to the parasite. Similar analyses have been performed for \textit{P. berghei} EXP2 by de Koning-Ward (unpublished), however, given how abundant the levels of EXP2 transcript are in the parasite, it has been difficult to obtain efficient knockdown of EXP2 expression for phenotypic analysis.

The ability to generate a \textit{P. berghei} TRX2 knockout reveals that at least in this species, TRX2 is a non-essential PTEX component, and therefore, very unlikely to make a suitable drug target. Nonetheless, the PbΔTRX2 parasites provided a valuable tool to dissect the role of PTEX in protein export as despite having a slower growth rate, the genetic modification was not lethal to the parasite. Whilst the export assays performed on the PbΔTRX2 line did not yield noticeable changes in the localization of endogenous proteins that are normally exported into punctate structures in the erythrocyte cytosol, the PbΔTRX2 parasites did have an overall reduction in global protein export even when delayed growth was taken into consideration. Given that the PbΔTRX2 parasites also exhibited a delay in their ability to export a GFP reporter, it is likely that loss of TRX2 leads to a reduced efficiency of overall export, although it cannot be ruled out that TRX2 may also play a role in exporting a particular subset of proteins that were not specifically investigated here. Importantly, when the export assays developed in this study were used to measure export deficiencies in the \textit{P. berghei} HSP101 inducible knockout parasites, Pbi101KD, the export results were very striking and consistent with anticipated levels of export when comparing disruption of
an essential PTEX component to a non-essential gene. Not only did Pbi101 KD have a much greater reduction in global parasite protein exported to the infected erythrocyte surface (Supp Figure 1), there were also clear differences between the export of individual PEXEL proteins, PbANKA_114540 and transmembrane domain positive, PbANKA_122900 as observed by IFA when exposed to different ATc regimes (Supp Figure 2). Moreover, knockdown of HSP101 also resulted in retention of a PEXEL-negative protein EMAP1 (PbANKA_083680) at the PV and this protein was not observed at its typical location at the erythrocyte membrane109 (Supp Figure 2). These assays, in combination with the assays undertaken on the *P. falciparum* PTEX150 knockout have provided the definite proof that PTEX is not only involved in the export of soluble cargo, but in the export of both PNEPs as well as transmembrane-domain containing proteins. Thus, PTEX is a central player in the protein export process and thus represents an Achilles’ heel of the parasite.

Despite export being drastically affected by PTEX disruption, limitations still exist in the experimental capabilities of phenotypically characterising the PTEX inducible knockouts as any change to PTEX that has a profound effect on protein export has dire consequences to parasite survival. Thus deciphering the function of individual PTEX components has not yet been definitely solved. Looking at other translocons that exist in nature has enabled predictions as to what they may be. Generally, whilst other translocons found in nature are unique, they share a number of unifying features, albeit whether they found in eukaryote organelles (eg. ER, mitochondria, chloroplasts or peroxisomes) or in prokaryotes, that include receptors to recognise cargo to be translocated, a membrane-spanning
component that provides passage through the membrane, energy (ATP, GTP or a membrane potential) to facilitate the translocation process as well as chaperones to unfold and refold cargo. Additionally, they comprise of components that are not essential for the translocon to operate, but which help to regulate activities such as translocon assembly or their configuration in an open or closed state, or the translocation of particular proteins under certain conditions. For example, genetic deletion of some members of the TOC complex of chloroplasts, have yielded minimal or no change in translocation phenotype. Deletion of Toc64, a translocon component that is peripherally associated through its interaction with Toc34\textsuperscript{206-208} in the chloroplasts of both in \textit{Physcomitrella patens} and \textit{Arabidopsis}\textsuperscript{209,210}, resulted in a change in the shape of chloroplast in moss. However the gene deletion in either species did not exhibit any changes to protein translocation\textsuperscript{209}.

The inability to knockout EXP2 is consistent with the original proposal by de Koning-Ward et al\textsuperscript{1} that this component forms the pore in the PVM. Analysis of chemical extraction fractions demonstrated that EXP2 associates most strongly with the integral membrane-associated fraction both in schizonts\textsuperscript{1} and ring-stage parasites\textsuperscript{176}, suggesting EXP2 is directly membrane-associated when PTEX presumably functions\textsuperscript{176}. EXP2 also displays a homo-oligomer confirmation indicative of its predicted pore-like structure\textsuperscript{176}. Nonetheless, evidence that EXP2 is the pore is still circumstantial and further work is required to dissect exactly how EXP2 associates with the membrane and if it is in fact indeed capable of forming pores through a membrane similar to HlyE, as previously proposed\textsuperscript{1} or some other mechanism.

Translocation of proteins in \textit{P. falciparum} requires energy\textsuperscript{157}. Inhibitors that
deplete ATP in the erythrocyte cytosol resulted in an arrest of secretion of glycophorin-binding protein (GBP). Addition of ATP reversed the phenotype and resulted in the translocation of GBP\textsuperscript{157}. In PTEX, the energy source is most likely provided by HSP101 activity based on its active AAA+ ATPase domain\textsuperscript{211} and the essentiality of this protein to both protein export and parasite survival is in keeping with this\textsuperscript{200}. ATPases are associated with diverse cellular functions and play essential roles in different cellular activities like proteolysis, membrane trafficking, cytoskeletal regulation, protein folding\textsuperscript{212}. They are found in all organisms and typically assemble into hexameric ring structures that are involved in energy dependent macromolecule remodeling\textsuperscript{180}. \textit{Plasmodium} HSP101 is also predicted to form a hexameric structure\textsuperscript{1} that undergoes conformational change after binding and hydrolysis of ATP\textsuperscript{213,214} to unfold protein cargo prior to translocation across the PVM. Thus, HSP101 likely functions in the opposite manner to the related ClpC chaperone\textsuperscript{211} (also termed HSP93), found in plant and algal chloroplasts, which docks onto Tic40/Tic110 in the TIC protein translocon system on the trans side of the membrane to ratchet proteins for import through the translocon\textsuperscript{208}.

Additionally, protein export in \textit{Plasmodium} parasites requires proteins to be unfolded\textsuperscript{156}. Thus, PTEX is similar to most other translocation machineries (eg, ER, chloroplast, mitochondria, and bacterial secretion systems) that require cargo to be in a loosely folded conformation so that they can cross the target membrane\textsuperscript{158,170,171}. In contrast, the thylakoidal system of bacteria or the twin Arg translocation (Tat) pathway, have the ability to transport tightly folded proteins\textsuperscript{215}. In other systems, misfolding, aggregation of cargo and the prevention of undesired interactions with other cellular proteins\textsuperscript{158}, relies on
the presence of chaperones to bind to the synthesized proteins and these
commonly depend on ATP hydrolysis for their activity\textsuperscript{158,216}. For example,
DnaK and DnaJ are well-established cytosolic chaperones in prokaryotes\textsuperscript{217}.
DnaK binds the protein to be translocated to prolong its translocation
conformation thus increasing the time for engagement of the signal sequence
with the translocon channel\textsuperscript{158,218,219}. Proteomic analysis of the \textit{Plasmodium}
vacuolar content revealed an abundance of chaperones\textsuperscript{198} and whilst
HSP101 may itself have a chaperone role, it may also require the assistance
of other proteins to keep the cargo in a competent state for export.

While the role of PTEX150 within PTEX remains unknown,
immunoprecipitation data suggests it interacts with both EXP2 and
HSP101\textsuperscript{176} and thus it may serve as a structural component. Keeping the
PTEX complex together would be consistent with the essential nature of
PTEX150 and the detrimental consequences that PTEX150 deletion has on
protein export. PTEX150 may serve as a platform for HSP101 to dock onto or
it may act as a receptor that receives cargo. The role of PTEX88 in the
complex also remains unknown. There are no distinguishing features in this
protein by which to predict function. Although it can be genetically deleted\textsuperscript{199},
parasites grow very poorly, indicating that whilst it is non-essential it is likely
to still make a significant contribution to PTEX function. Further assessment
of the PTEX88 gene deletion parasite line is required, with a specific focus on
protein export and how the gene deletion affects the stability and structure of
the PTEX complex.

Despite the fact the TRX2 could be genetically deleted and parasites could
be phenotyped to determine the consequence on protein export, virulence
and survival, at this point, it is still not possible to ascertain the specific role of TRX2. This will require the identification of its substrate protein/s. However, since TRX2 is a non-essential PTEX component, its role in PTEX is most likely to be regulatory. TRX2 could help to regulate the efficiency of PTEX by the formation of disulphide bonds within EXP2 or PTEX88 to maintain PTEX in an open or closed confirmation. Alternatively, it may regulate the export of cargo for export that contain disulphide bonds, by helping with unfolding prior to export. The export of proteins containing disulphide bonds, was not examined in this study, as the identification of such proteins are unknown in *P. berghei* and consequently there are no reagents available that specifically recognise these in *P. berghei*. It remains to be seen if deletion of TRX2 in *P. falciparum* is permissible and if the export of proteins such as STEVOR, RIFIN and PfEMP1, all of which contain disulphide bonds, is affected.

Using the export assays described in this study, functional evidence that PTEX is responsible for parasite protein export has been formally proven, especially with the HSP101 knockdown parasite line, in which export of both PEXEL and PNEPs was completely blocked\(^{200}\). This is direct evidence that the role of the PTEX molecular machine is to translocate all proteins destined for the host cell across the PVM into the host cytosol, and directly or indirectly, for parasite proteins expressed on the infected erythrocyte surface. However many questions about PTEX assembly and the mechanism of action still remain unanswered. PTEX has been described as being a five member complex, however like other translocons, it may actually comprise of more interacting partner proteins, some of which may only transiently interact with PTEX and thus may be difficult to identify. It also remains unknown how proteins destined for export reach PTEX or are specifically recognised by
PTEX, and whether there are multiple receptors that recognise distinct cargo. Along with this, chaperones are commonly required to refold the proteins translocated across a membrane and in *Plasmodium*, these may be either parasite derived or recruited from the host, however the identity of these have yet to be confirmed.

As PTEX provides a common portal for protein export, blocking its function with the use of drugs would block the export of hundreds of parasite proteins with diverse functions. From this study, the three essential components of PTEX, PTEX150, HSP101 and EXP2, would represent the best drug targets. However it is unknown which of these are in fact the most druggable. Affecting the ATPase activity may block HSP101, however inhibitors would need to be stringently selective against parasite HSP101 and not block the function of host HSP100 proteins, which are typically well conserved. For EXP2, the oligomerisation could be targeted to retard its ability to form a pore and the subsequent passage of exported proteins. It is less clear for PTEX150 how its function can be blocked with drugs, but like EXP2, this protein is completely unique to malaria parasites, and thus it is worthwhile to pursue drugs that target these proteins. Importantly, drug-targeting PTEX would be sufficiently novel to circumvent cross-resistance to existing anti-malaria drugs.
Chapter 7

Supplementary data
Supplementary Figure 1: Knockdown of *P. berghei* HSP101 blocks export of PEXEL and PNEP proteins.

a) Surface labeling of parasite antigens on Pbi101 KD parasites harvested between days 1 and 2 post infection from mice pretreated with ATc was substantially decreased compared with infected erythrocytes not exposed to ATc as measured by FACS. b) Staggered parasite sampling with ATc treatment given for 12 or 24 hrs (n=8; error bars represent s.e.m.; ***P < 0.001, using unpaired t-test). Boxes and whiskers delineate all data points, with whiskers indicating minimum and maximum values.
Supplementary Figure 2: Surface labeling of parasite antigens

**a)** Asynchronous Pbi101 KD parasites grown to high parasitemia followed with ATc treatment for either 12 or 24 h (n = 8; error bars represent s.e.m.). Representative IFA of 100 Pbi101 KD intraerythrocytic stages, showing that exposure to ATc blocks export of PEXEL (top and middle panels) and PNEP (bottom panel) proteins. Yellow bar in all diagrams, signal sequence; red bar, transmembrane domain; black bar, glycosylphosphatidylinositol anchor. DIC, differential interference contrast. **b)** Expression of MSP8 is not affected by ATc treatment. Scale bars, 5μm.
Chapter 8

Appendix
The Exported Protein PbCP1 Localises to Cleft-Like Structures in the Rodent Malaria Parasite Plasmodium berghei

Silvia Haase, Eric Hanssen, Kathryn Matthews, Ming Kalanon, Tania F. de Koning-Ward

1 School of Medicine, Deakin University, Waurn Ponds, Victoria, Australia; 2 Electron Microscopy Unit, Biod2 Institute, University of Melbourne, Parkville, Victoria, Australia

Abstract

Protein export into the host red blood cell is one of the key processes in the pathobiology of the malaria parasite Plasmodium falciparum, which extensively remodels the red blood cell to ensure its virulence and survival. In this study, we aimed to shed further light on the protein export mechanisms in the rodent malaria parasite P. berghei and provide further proof of the conserved nature of host cell remodeling in Plasmodium spp. Based on the presence of an export motif (KxLe/Q/D)x termED PXEL (Plasmodium export element), we have generated transgenic P. berghei parasite lines expressing GFP chimera of putatively exported proteins and analysed one of the newly identified exported proteins in detail. This essential protein, termed PbCP1 (P. berghei Cleft-like Protein 1), harbours an unusual PXEL motif (KxLe/Q/D)x termED PXEL and is further characterized by two predicted transmembrane domains (TMDs) in the C-terminal end of the protein. We have functionally validated the unusual PXEL motif in PbCP1 and analyzed the role of the TMD region which is required to recruit PbCP1 to discrete membranous structures in the red blood cell cytosol that have a constricted, vesiculotubular morphology by electron microscopy. Importantly, this study reveals that rodent malaria species also induce modifications to their host red blood cell.

Introduction

The human malaria parasite Plasmodium falciparum exports approximately three hundred proteins into its host red blood cell (RBC) [1] leading to a number of morphological alterations to the cell such as increased rigidity and adherence [8,16]. Similar ultrastructural cleft-like structures are found in RBCs infected with rodent malaria parasites [13]. Transmembrane domains are present in the host cell cytoskeleton including the Mammalian clefts, which have been implicated to be involved in sorting and trafficking of virulence proteins in RBC [14]. The presence of these structures is essential for parasite development and survival [15]. The parasite has evolved to remodel its cytoskeleton and to exploit the host cell cytoskeleton for efficient export of proteins and to ensure parasite replication. The parasite has evolved to use its own trafficking machinery to export its proteins from the endoplasmic reticulum (ER), including its own boundaries across the parasitophorous vacuole (PV) and parasitophorous vacuole membrane (PVM) into the host RBC. Passage of most exported proteins across the parasite-host cell interface is mediated by a pentameric amino acid motif (KxLe/Q/D)x termED PXEL (Plasmodium export element) or HT (host targeting) motif, which is located downstream of an N-terminal signal peptide [9,16]. Cleavage of this motif selectively enables these proteins to cross the parasite-host cell boundary through a putative Plasmodium transmembrane exported proteins (PTEX) [11,12]. While both the PXEL motif and PTEX are conserved amongst Plasmodium spp [11,2], little is known about protein export and host cell modifications in other malaria parasite species as previous studies have focused predominantly on P. falciparum. However, to investigate the functional significance of protein export in malaria pathogenesis, it is essential to study this in the context of the host by using Plasmodium species for which animal models exist. Recent studies have provided the first evidence of PXEL-mediated export of P. falciparum protein in the rodent malaria parasite P. berghei and of export of rodent parasite proteins is a PEXEL-dependent and independent manner [13,15]. Moreover, the finding that P. berghei ANKA parasites indeed sequester [16] suggest common host cell remodeling strategies may be utilized amongst Plasmodium spp. to ensure parasite virulence and survival.

Thus, in order to gain a deeper insight and understanding into the evolution of protein export and its importance in malaria pathogenesis, we aimed to further evaluate the rodent "response" by identifying novel exported proteins in the rodent malaria parasite P. berghei and the mechanisms by which they are exported.
The *Plasmodium* translocon of exported proteins (PTEX) component thioredoxin-2 is important for maintaining normal blood-stage growth

Kathryn Matthews,¹ Ming Kalanon,¹ Scott A. Chisholm,¹ Angelika Sturm,² Christopher D. Goodman,¹ Matthew W. A. Dixon,³ Paul R. Sanders,⁴ Thomas Nebi,¹ Fiona Fraser,¹ Silvia Haase,¹¹ Geoffrey I. McFadden,⁷ Paul R. Gillson,⁷ Brendan S. Crabbe and Tania F. de Koning-Ward⁸

¹School of Medicine, Deakin University, Waurn Ponds, Vic. 3216, Australia.
²School of Botany, University of Melbourne, Melbourne, Vic. 3010, Australia.
³Department of Biochemistry and Molecular Biology, Biod2 Institute, Melbourne, Vic. 3010, Australia.
⁴Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Vic. 3004, Australia.
⁵Infection and Immunity Division, The Walter and Eliza Hall Institute, Melbourne, Vic., 3052, Australia.

Summary

*Plasmodium* parasites remodel their vertebrate host cells by translocating hundreds of proteins across an encasing membrane into the host cell cytosol via a putative export machinery termed PTEX. Previously PTEX150, HSP101 and EXP2 have been shown to be bona fide members of PTEX. Here we validate that PTEX88 and TRX2 are also genuine members of PTEX and provide evidence that expression of PTEX components are also expressed in early gametocytes, mosquito and liver stages, consistent with observations that protein export is not restricted to asexual stages. Although amenable to genetic tagging, HSP101, PTEX150, EXP2 and PTEX88 could not be genetically deleted in *Plasmodium berghei*. In keeping with the obligatory role this complex is postulated to have in maintaining normal blood-stage growth. In contrast, the putative thioredoxin-like protein TRX2 could be deleted, with knockout parasites displaying reduced grow-rates, both in vivo and in vitro, and reduced capacity to cause severe disease in a cerebral malaria model. Thus, while not essential for parasite survival, TRX2 may help to optimize PTEX activity. Importantly, the generation of TRX2 knockout parasites that display altered phenotypes provides a much-needed tool to dissect PTEX function.

Introduction

Malaria remains a global health burden, causing approximately 660,000 deaths and a further 220 million clinical cases annually (World Health Organization, 2012). The clinical symptoms of malaria are associated with the stage when *Plasmodium* parasites reside within red blood cells (RBCs), with *Plasmodium falciparum* responsible for the largest proportion of human symptomatic infection. The pathogenicity of *P. falciparum* is attributable to its remarkable ability to radically remodel its host RBC; a process involving a number of sophisticated mechanisms that lead to the trafficking of hundreds of parasite proteins beyond its encasing parasitophorous vacuole membrane (PVM), with consequential structural and biochemical changes to the host cell (for a review see Maer et al., 2009; Haase and de Koning-Ward, 2010). The diverse roles these exported proteins play include facilitating nutrient and solute exchange as well as preventing adhesion proteins, particularly PREM1, on the infected RBC surface (Crabb et al., 1997; Staines et al., 2006; Ngalurugoi et al., 2011). The latter facilitates binding of the infected RBC to the microvasculature, thereby preventing their clearance from the spleen and additionally leading to the pathologies associated with *Plasmodium* infection (David et al., 1983; Duffy and Fried, 2003; Haldar and Mohandas, 2007).

The discovery of a conserved pentamic peptide PEXEL motif that mediates protein export into the host RBC has enabled the ‘exportome’ of malaria parasites to be defined (Hiller et al., 2004; Martin et al., 2004). While *P. falciparum* is predicted to export between 300 and 400 proteins, comparison of the orthologous proteins in other *Plasmodium* spp. revealed they possess much smaller exportomes (Martin et al., 2004; Sargeant et al., 2006; van Ooij and Haldar, 2007; van Ooij et al., 2008; Pick et al., 2011; Boddey et al., 2013). One plausible explanation for the larger exportome in *P. falciparum* is the expansion of protein families in this species, including those that contain
PTEX is an essential nexus for protein export in malaria parasites

Brendan Ellsworth1,2,3, Kathryn Matthews1,2, Catherine Q. Neel1, Ming Kalamas3, Sarah C. Charnaud4, Paul R. Sanders5, Scott A. Chisolm1, Natalie A. Cournats1,8, Philip J. Straw6, Paco Pinto6, Jo-Ann Charette1, Mauro F. Azevedo6, Stephen J. Rogers6, James G. Beeson7,2, Brendan S. Crabbe4,6,8, Paul R. Gilbert4,6,8 & Tania F. de Koning-Ward1,8

During the blood stages of malaria, several hundred parasite-encoded proteins are exported beyond the double-membrane barrier that separates the parasite from the host cell cytoplasm8. These proteins have a variety of roles that are essential to virulence or parasite growth7. There is keen interest in understanding how proteins are exported and whether common machineries are involved in trafficking the different classes of exported proteins9,10. One potential trafficking machine is a protein complex known as the Plasmaform transnoum of exported proteins (PTEX)11. Although PTEX has been linked to the export of one class of exported proteins8,11,12, there has been no direct evidence for its role and scope in protein translocation. Here we show, through the generation of two parasite lines defective for essential PTEX components (HSP101 or PTEX150), and analysis of a line lacking the non-essential component TRYX (ref. 13), greatly reduced trafficking of all classes of exported proteins beyond the double membrane barrier enveloping the parasite. This includes proteins containing the PEXEL motif (RexExE/G/Q)14,15 and PEXEL-negative exported proteins (PNEP)16. Moreover, the export of proteins destined for expression on the infected erythrocyte surface, including the major virulence factor PEMP1 in Plasmodium falciparum, was significantly reduced in PTEX knockdown parasites. PTEX function was also essential for blood-stage growth, because even a modest knockdown of PTEX components had a strong effect on the parasite’s capacity to complete the erythrocyte cycle both in vitro and in vivo. Hence, as the only known nexus for protein export in Plasmodium parasites, and an essential conserved machine, PTEX is a prime drug target.

To address the role of PTEX in protein export directly, we examined parasite lines defective in PTEX components for their capacity to translocate exported proteins. Two PTEX components, TRYX and PTEX88 (Fig. 1a), have auxiliary roles in PTEX function, because their deletion results in a substantial parasite growth defect13,17. We therefore assumed that PTEX function is suboptimal in these lines. Here we show that surface expression of parasite antigens was substantially reduced in Plasmodium berghei TKD-deficient parasite18 (Extended Data Fig. 1), which is consistent with a role for PTEX in protein export. To perturb PTEX function more fully, conditional mutants of two essential PTEX components, HSP101 and PTEX150 (refs 10, 12, 13), were generated. These proteins are synthesized in late schizonts and early ring stage and reside in the parasitophorous vacuole membrane for the remainder of the erythrocytic cycle19.

For HSP101, we generated a p. berghei line, Pho101 KD, harbouring HSP101 under the transcriptional control of an inducible transactivator (ATC)-regulated transactivator element10 (Extended Data Fig. 2a). The growth of Pho101 KD parasites was specifically sensitive to treatment with ATC (Fig. 1d,e). The Pho101 KD line grew poorly in mouse-pre-exposed to ATC-24h before infection (Fig. 1f,g). Pho101 KD parasites were disrupted in the absence of ATC could be rescued by ATC was added at day 4 (Fig. 1d, middle panel, and Extended Data Fig. 2b). As expected, the growth of parental P. berghei ANKA parasites was unaffected by the presence of ATC (Fig. 1d, bottom panel).

To examine the growth effect in more detail, purified ring-stage Pho101 KD parasites were injected into mice pre-exposed to ATC, then isolated 24h later and cultured in vitro with ATC (Fig. 1e). As expected, parasites invaded erythrocytes in the mice and developed normally into ring stages (Fig. 1e, 24h time point). However, parasites appeared morphologically abnormal by the 34h time point and were incapable of developing into schizonts by the 46h time point, unlike Pho101 KD parasites not exposed to ATC. Asynchronous Pho101 KD ring-stage parasites cultured in vitro for 16h in the presence of ATC demonstrated a threefold to fold decrease in hep101 messenger RNA in schizont stages (Fig. 1f) and a 65%–89% knockdown of HSP101 protein by the 24h time point in Fig. 1e relative to the loading control protein EXP32 and Msp8 (Fig. 1g).

To examine whether HSP101 knockdown affected protein export we assessed whether asynchronous Pho101 KD parasites harvested from ATC-preexposed mice at the time point indicated by the gray bar in Fig. 1d displayed surface-expressed antigens. Pho101 KD parasites showed a striking reduction in parasite-encoded surface antigens compared with parasites grown in the absence of ATC (Fig. 2a). In an alternative approach, Pho101 KD parasites grown to a higher parasitemia (~10%) in the absence of ATC were subsequently treated with ATC for either 12 or 24h before analysis. Given the asynchronous of the infection, some parasites would already have transcribed HSP101 before ATC treatment commenced; consistent with this, surface expression was reduced in a manner dependent on the duration of ATC exposure (Fig. 2b).

We also assessed the export of individual proteins by immunofluorescence assay (IFA) using specific antibody reagents against three different exported proteins. Two of these proteins, PbAKNA, 114540 and PbAKNA, 122900, contain the PEXEL motif (RexExE/G/Q) and localize to punctate structures in the erythrocyte cytoplasm14. C. K. Mointira, B. Naissant, A. Coppa, L. Bennett, E. Aime, B. Frielse-Fayard, C. J. Issae, L. Coppens, P. Stamia and T. J. Templeton, personal communication), whereas PbAKNA, 083680 (EMAP1) is a PNP that localizes to the erythrocyte membrane16. In each case, a striking blockage of protein export was observed in Pho101 KD parasites exposed to a variety of different ATC treatment regimes; this included Pho101 KD harvested from rice at the time points represented by the gray bar and asterisks in Fig. 1d (Fig. 2c and Extended Data Fig. 3a, respectively) and in Fig. 1e (Fig. 2c and Extended Data Fig. 3a, respectively). As expected, morphology normal ring-stage parasites examined at the 24 and 24h time points in Fig. 1e, observable export of PbAKNA, 114540 and PbAKNA, 083680 could only be detected in 7 of 131 and 5 of 100 parasites, respectively, whereas none of 100 parasites visibly expressed PbAKNA, 122900. In contrast, almost equivalent numbers of Pho101 KD parasites grown with or without ATC (80% and 88% expression, respectively) expressed Msp98.

1National Institute of Medical Research and Public Health, Melbourne, VIC, Australia. 2Vanderbilt University, Nashville, TN, USA. 3Monash University, Melbourne, VIC, Australia. 4National Centre for Genetic Engineering and Biotechnology (NCGEB), Pathum Thani 12220, Thailand. 5The University of Georgia, Athens, GA, USA. 6The University of Melbourne, Parkville, VIC, Australia. 7The University of Melbourne, Parkville, VIC, Australia. 8These authors contributed equally to this work.

Cite this article as: PTEX is an essential nexus for protein export in malaria parasites, Nature 511, 187 (2014) doi:10.1038/nature1355.
Chapter 9

References


51 Rogerson, S. J., Novakovic, S., Cooke, B. M. & Brown, G. V. *Plasmodium falciparum*-infected erythrocytes adhere to the


100 Pachlatko, E. et al. MAHRP2, an exported protein of *Plasmodium falciparum*, is an essential component of Maurer's cleft tethers. *Molecular Microbiology* 77, 1136-1152 (2010).


113 Akinyi, S. et al. A 95 kDa protein of *Plasmodium vivax* and *P. cynomolgi* visualized by 3-D tomography in the caveola-vesicle complexes (Schüffner's dots) of infected erythrocytes is a member of the PHIST family. *Molecular Microbiology* **84**, 816-831 (2012).


152 Spielmann, T. et al. A cluster of ring stage - specific genes linked to a locus implicated in cytoadherence in *Plasmodium falciparum* codes for


