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Analysis of Protein Translocation at the Interface between the Malaria Parasite *Plasmodium falciparum* and its Host Cell

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To my parents

Contents

Abstract

Malaria remains a major infectious disease with annually more than 200 million cases causing more than 400.000 deaths worldwide. It is caused by blood stages of *Plasmodium* parasites. This phase of the parasite's life cycle is characterised by the multiplication of the pathogen inside red blood cells (RBCs), leading to the destruction of the host cell and the release of new parasites that invade new RBCs. Inside its host cell, the pathogen resides in a membrane-bounded compartment termed the parasitophorous vacuole (PV).

In order to create an optimal environment for growth and replication, the parasite modifies its host cell by exporting a variety of proteins into the infected erythrocyte. Some of these exported proteins lead to an enhanced nutrient uptake into the RBC and increase the rigidity of the cytoskeleton while others confer adhesion to the endothelium and undergo antigenic variation to evade the host immune system. A family of these exported surface antigens is responsible for many of the severe complications of malaria. Protein export into the infected RBC is thus crucial for both the development and the virulence of *Plasmodium* parasites.

Exported proteins need to cross the parasite's plasma membrane (PPM) as well as the PV membrane (PVM) in order to reach their destination. A recently identified protein complex called PTEX which is located at the inner surface of the PVM is thought to translocate exported proteins into the host cell. Several components of PTEX were shown to be essential for protein export. However, it is still unclear how PTEX facilitates the export of substrates that comprise remarkably different classes of proteins. It is also not known how transmembrane proteins cross the PPM before reaching PTEX.

In this thesis, we utilise parasite proteins fused to conditionally foldable domains to demonstrate that different classes of exported proteins (soluble proteins as well as transmembrane proteins with or without canonical export signal) require unfolding in order to be successfully exported. We provide evidence that the export pathways of those proteins converge at the same unfolding-dependent step, and that this step is most likely located at the inner surface of the PVM, the site of PTEX activity. Lastly, we provide evidence that the export of some transmembrane proteins requires an additional unfolding-dependent step at the PPM. These findings contribute to our understanding of the sequence of trafficking events that exported proteins undergo at the interface between *Plasmodium* parasites and their host cell.

Kurzdarstellung

Mit jährlich über 200 Millionen Erkrankten und über 400.000 Todesopfern stellt Malaria weiterhin eine der bedeutsamsten Infektionskrankheiten weltweit dar. Die Krankheit wird durch Blutstadien von Parasiten der Gattung *Plasmodium* ausgelöst. Während dieses Abschnittes im Lebenszyklus des Parasiten vermehrt sich dieser in infizierten Erythrozyten, was schließlich zur Zerstörung der Wirtszelle und zur Freisetzung neuer Parasiten in die Blutbahn führt. In seiner Wirtszelle befindet sich der Krankheitserreger dabei in einem von einer Membran begrenzten Kompartiment, welches als parasitophore Vakuole (PV) bezeichnet wird.

Um optimale Wachstumsbedingungen zu schaffen, modifiziert der Parasit seine Wirtszelle durch den Export einer Vielzahl von Proteinen in den infizierten Erythrozyten. Einige dieser exportierten Proteine führen zu einer erhöhten Nährstoffaufnahme in die infizierte Zelle und vermindern die Elastizität des Zytoskeletts, während andere zur Adhäsion des Erythrozyten am Gefäßendothel führen und durch Antigenvariation zur Umgehung der Immunantwort beitragen. Eine Familie solcher exportierter Oberflächenantigene ist auch für viele der schweren Komplikationen der Malaria verantwortlich. Der Export von Proteinen in infizierte Erythrozyten ist demnach ein entscheidender Mechanismus sowohl für das Wachstum als auch für die Virulenz von *Plasmodium* Parasiten.

Um an ihren Zielort zu gelangen, müssen exportierte Proteine die Plasmamembran des Parasiten (PMP) und die PV-Membran (PVM) überqueren. Es wird angenommen, dass PTEX, ein Proteinkomplex an der luminalen Seite der PVM, exportierte Proteine in die Wirtszelle translozierte. Es wurde gezeigt, dass mehrere PTEX-Komponenten essentiell für den Export von Proteinen sind. Es ist jedoch unklar, wie PTEX den Export von Substraten mit teilweise sehr unterschiedlichen Eigenschaften ermöglicht. Auch ist bislang unbekannt, durch welchen Mechanismus exportierte Transmembranproteine über die PMP gelangen.

In dieser Dissertation untersuchen wir den Exportmechanismus mithilfe von parasiteneigenen Proteinen, die mit Domänen modifiziert wurden, welche unter bestimmten Bedingungen an der Entfaltung gehindert werden können. So zeigen wir, dass verschiedene Klassen exportierter Proteine entfaltet werden müssen, um exportiert werden zu können. Zu den Klassen untersuchter Proteine gehören solche mit oder ohne bekanntem Exportsignal, lös-

liche Proteine sowie Transmembranproteine. Weiterhin zeigen wir, dass die Exportwege verschiedener Substrate an einem Punkt zusammentreffen, an dem die Polypeptide entfaltet werden müssen, und dass dieser Punkt an der luminalen Seite der PVM liegt, wo auch PTEX aktiv ist. Zuletzt liefern wir Hinweise darauf, dass einige exportierte Transmembranproteine zusätzlich bereits an einem früheren Punkt entfaltet werden müssen, um die PMP zu überqueren. Diese Ergebnisse tragen zu unserem Verständnis der Prozesse bei, die während des Blutstadiums von Plasmodien den Export von Proteinen über die Berührungsfläche zwischen Parasit und Wirtszelle ermöglichen.

Abbreviations

$\times g$	times g (standard gravity of Earth, 9.80665 $\frac{m}{s^2}$)
°C	degrees Celsius
µm	micrometre
µg	microgram
µl	microlitre
*Pf*EMP1	*P. falciparum* erythrocyte membrane protein 1
AA	amino acids
ACT	artemisinin-based combination therapy
BPTI	bovine pancreatic trypsin inhibitor
BSD	Blasticidin S
Clag3	cytoadherence-linked asexual protein 3
Clp	caseinolytic protease
DALY	disability-adjusted life year
DAPI	4',6-diamidino-2-phenylindole
DHFR	dihydrofolate reductase
DNA	dexoyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTG	Deutsche Gesellschaft für Tropenmedizin und Internationale Gesundheit
EDTA	ethylenediaminetetraacetate
EPIC	Exported Protein-Interacting Complex
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
EtOH	ethanol
EXP2	exported protein 2
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
h	hour(s)
HCl	hydrochloric acid
hDHFR	human dihydrofolate reductase

HF	high fidelity
HRP	horseradish peroxidase
HSP	heat shock protein
IgG	Immunoglobulin G
iRBC	infected red blood cell
KAHRP	knob-associated histidine-rich protein
LB	lysogeny broth
LM	light microscopy
M	molar
MAHRP1	Maurer's Clefts-associated histidine-rich protein 1
MC	Maurer's Clefts
mCherry	monomeric Cherry, red fluorescence tag
mDHFR	murine dihydrofolate reductase
mg	milligram
min	minute(s)
ml	millilitre
mM	milimolar
MS	mass spectrometry
MSRP6	merozoite surface-related protein 6
OD	optical density
ON	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEXEL	plasmodial export element
PK	proteinase K
PMFS	phenylmethylsulfonylfluoride
PNEP	PEXEL-negative exported protein
PPM	parasite plasma membrane
PSAC	*Plasmodium* surface anion channel
PTEX	Plasmodial Translocon of Exported Proteins
PTP1	PfEMP1-trafficking protein 1
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
RBCs	red blood cells
RDT	rapid diagnostic test
RE	restriction endonuclease
REX2	ring-exported protein 2

REX3	ring-exported protein 3
rif	repetitive interspersed fragment
RKI	Robert Koch Institut
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
s	second(s)
SDS	sodiumdodecylsulfate
SELMA	symbiont-derived ERAD-like machinery
SP	signal peptide
STEVOR	sub-telomeric open reading frame
TE	tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate
TEMED	tetramethylenediamine
TIM	translocon of the inner membrane
TM	transmembrane
TMD	transmembrane domain
TOM	translocon of the outer membrane
TRIS	tris(hydroxymethyl)aminomethane
TVN	tubulovesicular network
U	unit of enzyme activity
vol.	volume
WHO	World Health Organisation
WR	WR99210
Å	Angström

Chapter 1

Introduction

1.1 *Plasmodium falciparum* as a Pathogen

1.1.1 Epidemiology and Impact on Global Health

Since 2008, the World Health Organisation (WHO) issues its annual World Malaria Report as a collection of data from routine surveillance in endemic countries and a selection of other sources (WHO 2016). Thus, this report represents a comprehensive resource of data on malaria epidemiology and of current worldwide efforts and developments in disease control.

Malaria is endemic mainly in sub-tropical and tropical areas around the globe and half of the world's population lives at risk of the disease (WHO 2017c) (Figure 1.1). In 2015, malaria transmission occurred in 91 different countries (WHO 2016). As of today, the incidence of malaria is estimated at 212 million cases with 429.000 deaths worldwide (WHO 2016). An estimated 99% of those deaths are attributed to *Plasmodium falciparum* (*P. falciparum*, see section 1.2.1). 90% of malaria cases and 92% of deaths are estimated to occur in Sub-Sahara Africa and 70% of deaths to occur in children under the age of five (WHO 2016). Other subpopulations that are disproportionally affected by severe malaria include pregnant women, individuals with HIV/AIDS and travellers previously unexposed to the pathogen (WHO 2017c).

A measure to assess the socioeconomic impact of a disease is the disability-adjusted life year (DALY). This measure takes into account the years of life lost due to premature death as well as due to a state of impaired health, i.e. disability (Kassebaum, Arora, Barber, et al. 2016). While the definition of DALY only copes with certain dimensions of disease, it provides a standardized tool to compare the burden of different diseases on a society. Malaria was the largest contributor to the total burden of disease in many countries of Sub-Saharan

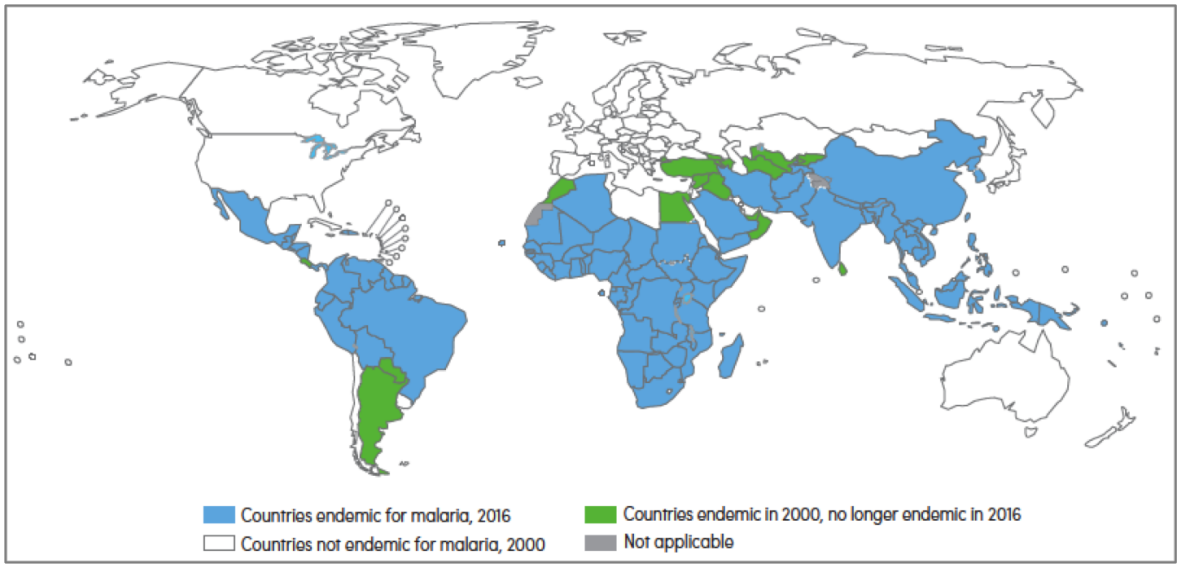

Figure (1.1): Malaria endemicity. Comparison of malaria-endemic countries, 2000 v. 2016. Source: (WHO 2016).

Africa and ranked 14th globally as a cause for DALYs (Kassebaum, Arora, Barber, et al. 2016).

International efforts to control the disease include prevention (e.g. vector control, distribution of mosquito nets, chemoprophylaxis, education) and treatment (e.g. improved diagnostic tools, new antibiotic regimens, improved health infrastructure) and have yielded some success over the course of the last two decades. For example, the global incidence of malaria decreased by an estimated 41% and the mortality decreased by an estimated 62% between 2000 and 2015. Furthermore, several countries have recently been declared malaria-free by the WHO (Figure 1.1).

However, as the numbers above indicate, malaria remains one of the most important infectious diseases worldwide as a major contributor to illness, death, and overall burden of disease in many countries.

The "Global Technical Strategy for Malaria 2016-2030", adopted by the World Health Assembly in 2015, has the goal of a global reduction of malaria incidence of 90% until 2030 and defines a set of milestones to be achieved by the international community in this period (WHO 2015a). Towards this end, the comprehensive implementation of existing effective measures (see section 1.1.2) will be as important as the development of other new strategies, to which a better understanding of the pathogen's biology may contribute.

1.1.2 Clinical Aspects of Malaria

Five different *Plasmodium* species are known to infect humans (see section 1.2.1 and the clinical presentation of Malaria may differ greatly depending on the pathogen, especially with regard to incubation period, fever periodicity and severeness of the disease. However, the focus of this introduction will be on the most important species *P. falciparum*. A summary of clinical aspects of *falciparum*-malaria will be given below. Exhaustive information on this subject as well as guidelines for the diagnosis and treatment of this disease are provided by global and national healthcare authorities (e.g. the WHO, the Deutsche Gesellschaft für Tropenmedizin und Internationale Gesundheit (DTG) and the Robert Koch Institut (RKI) for Germany).

Pathophysiology

The different stages of the *Plasmodium* life cycle are described in detail in section 1.2.2. After the transmission of sporozoites through the bite of an infected mosquito, the parasites' migration into hepatocytes and its intrahepatic development is asymptomatic. The unspecific, flu-like symptoms of malaria develop after the transition into the parasites' blood stage which is marked by continuous asexual replication. The rupture of blood stage schizonts is coupled with the release of merozoites and cellular debris into the blood stream, which induces the release of inflammatory cytokines and triggers the body's immune reaction (Miller et al. 2002; Bartoloni and Zammarchi 2012). The erythrocytic half-life is reduced by invading parasites and accelerated clearance by the spleen, leading to anaemia and jaundice (Buffet et al. 2011). About 1-2% of infections lead to severe malaria (Wassmer et al. 2015) (see *Clinical Presentation* below).

The pathophysiology of severe malaria, which is caused almost exclusively by *P. falciparum*, remains to be fully understood. Sequestration of infected erythrocytes into the microvasculature mediated by surface antigens such as *Pf*EMP1 (Hviid and Jensen 2015) (*P. falciparum* erythrocyte membrane protein 1), leading to the obstruction of small blood vessels, tissue hypoxia and local inflammation, is thought to play a critical role in the development of cerebral malaria and acute kidney injury (Miller et al. 2002; WHO 2014; Wassmer et al. 2015). Severe anemia most often affects children where it is the result of repeated infections without full recovery and the reduced erythrocyte half-life (WHO 2014). Lactic acidosis is attributed to a combination of tissue hypoxia in organs affected by microvascular obstruction, the parasite metabolism and a reduced clearance of lactic acid in the liver and kidneys (WHO 2014). Hypoglycemia is a result of an impaired hepatic gluconeogenesis and the accelerated peripheral metabolism by the febrile host and may lead to ketoacidosis (WHO

2014). The cause for pulmonary oedema is unclear. This complication often develops after the initiation of antimalarial treatment and involves local inflammation and capillary leakage (WHO 2014). Finally, severe jaundice is the result of haemolysis and hepatic dysfunction (WHO 2014).

Clinical Presentation

The symptoms of *falciparum*-malaria follow an incubation period of at least 7 days up to several months and may initially "comprise headache, lassitude, fatigue, abdominal discomfort and muscle and joint aches, usually followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise" (WHO 2015b).

While the fever chart in non-*falciparum* malaria often shows typical periodic peaks recurring every 48h (tertian fever, *P. vivax* and *P. ovale*) or 72h (quartan fever, *P. malariae*), *falciparum*-malaria normally lacks such periodicity (Bartoloni and Zammarchi 2012).

Signs for severe malaria, caused by *P. falciparum* and, in most cases, developing under insufficient treatment, include: "coma (cerebral malaria), metabolic acidosis, severe anaemia, hypoglycaemia, acute renal failure or acute pulmonary oedema" (WHO 2015b) (see *Pathophysiology*, above).

Diagnosis

The early symptoms of malaria are unspecific signs of infection and usually do not allow to rule out other causative agents. Depending on the setting and parasite species, Plasmodia may persist in a patient's body without causing symptoms for months or years. Thus, it is important for clinicians in non-endemic areas to investigate the possibility of past exposure to the pathogen (DTG 2016; RKI 2015). After the suspicion of Malaria is raised based on clinical criteria adjusted to the endemic situation of an area, the most important tool to establish the diagnosis remains the examination of Giemsa stained thick and thin blood smears by light microscopy (LM) (WHO 2015b; DTG 2016; RKI 2015). In addition, during the last 20 years the use of rapid diagnostic tests (RDTs) for malaria has been established as a supportive means of diagnosis as well as an alternative to LM in cases where LM is not available (WHO 2011a; WHO 2011b). RDTs recommended by the WHO are lateral flow assays that detect parasite antigens in small quantities of patient's blood with the help of labeled antibodies (WHO 2011a).

While other laboratory methods for the diagnosis of malaria such as serological tests and PCR are available, they currently play a secondary role in clinical practice as they are usually either more elaborate and expensive or less sensitive than LM and RDTs (Tangpukdee et al.

2009). However, it is noteworthy that PCR is considered to be the most sensitive and specific test for malaria available and may be useful to detect cases with very low parasitaemia or to discern the *Plasmodium* species (Tangpukdee et al. 2009; DTG 2016).

LM is considered to have a detection threshold of 100-500 parasites per microliter of blood (equivalent to 0.002% to 0.01% parasitaemia) under field conditions (10-fold lower under optimal conditions) compared to 1-5 parasites per microliter (below 0.0001%) for PCR. RDTs are considered to have a wide range of sensitivity, depending on the *Plasmodium* species and other factors, and perform worse than LM in cases with low parasitaemia and non-*falciparum* malaria (Tangpukdee et al. 2009; Mathison and Pritt 2017).

Treatment

The first-line treatment of uncomplicated malaria today is the artemisinin-based combination therapy (ACT) consisting of one artemisinin-derivative (artemether, artesunate or dihydroartemisinin) paired with another antimalarial. Artemisinin-based drugs rapidly eliminate both asexual and sexual blood stages, providing an additional benefit in the prevention of transmission. The partner drug has a longer plasma half-life, thus allowing for an overall shorter period of treatment and preventing the development of resistance to artemisinin. Among the five ACT partner drugs recommended by the WHO, four are thought to interfere with different steps of the parasite's heme detoxification (lumefantrine, amodiaquine, mefloquine and piperaquine), while the combination of sulfadioxine and pyrimethamine acts as a plamodial antifolate. Chloroquine plays a subordinate role in the treatment of uncomplicated non-*falciparum* malaria when the probability of resistance is very low. Further, quinine is used for the treatment of uncomplicated malaria in pregnant women during their first trimester (WHO 2015b).

Severe malaria is treated with parenteral antimalarials such as artesunate (first-line), artemeter or quinine over a minimum of 24h, followed by 3 days of ACT. Each suspected case of severe malaria has to be considered a medical emergency and requires the availability of intensive care for adequate treatment (WHO 2013b; WHO 2015b).

Resistance

Since the first introduction of effective antimalarial compounds, the deployment of every new drug has been accompanied by the developement and spread of resistance among parasites. Today, as a result, *P. falciparum* is widely resistant to once effective antimalarials such as chloroquine and sulfadoxine-pyrimethamine, largely reducing their impact to partner drugs in ACT, although the incidence of drug resistance may vary dramatically in different geo-

graphic regions. The WHO does not recommend monotherapies of any compound for the treatment of uncomplicated malaria to slow down the development of resistances (WHO 2010). Recently, strains of *P. falciparum* showing reduced susceptibility to artemisinin have been identified in South East Asian regions that seem to favour the developement of resistance through environmental factors (Dondorp et al. 2009; Phyo et al. 2012; Miotto et al. 2013). This alarming discovery highlights the importance of continued efforts in the developement of antimalarial strategies, as a spread of artemisinin resistance to Africa would seriously jeopardize this century's progress in containing the disease (WHO 2010; WHO 2016).

Prevention and containment

Available measures for the prevention and containment of malaria currently comprise chemoprophylaxis and vector control. Vaccines may provide a complementary tool to control the disease, but despite strong efforts, it remains uncertain whether the ultimate goal to develop an efficient vaccine capable to reliably provide lasting immunity can be obtained any time soon (WHO 2015b; WHO 2016).

Chemoprophylaxis may be used by travellers to endemic regions or by special risk groups to prevent malaria and is achieved by maintaining therapeutic plasma concentrations of antiparasitic drugs over a period of time. Antimalarials used for this purpose are non-artemisinin agents with a long plasma half-life and need to be chosen considering the regional resistance patterns (WHO 2015b) .

The most effective means of vector control are indoor residual spraying of insecticides (Pluess et al. 2010) and sleeping under insecticide-treated nets (Lengeler 2004). The impact of these two vector-control measures on malaria prevalence in Sub-Saharan Africa is considered to be substantial (Bhatt et al. 2015). Larval source management can complement these measures and subsumes interventions that target *Anopheles* larvae in their habitats (WHO 2013a). Such measures include modifactions and manipulations of the environment in which the larvae develop, for example the drainage of surface water or the introduction of natural predators.

Vaccine developement has been a main target of malaria research for several decades without any breakthrough success. In recent years several candidates have reached clinical or pre-clinical stages of development (WHO 2017b). To date, only one compound, termed RTS,S/AS01, has completed phase 3 testing and received a positive assessment by the European Medicines Authority (WHO 2016; WHO 2017a). However, immunity induced by this candidate is incomplete and short-lived compared to other vaccines and the overall outcome so far has been sobering. Thus, RTS,S/AS01 should be considered a complementary mea-

sure to control malaria, the effectiveness of which will be assessed in further pilot studies - and a step in the process towards more effective vaccines (WHO 2017a).

1.2 The Biology of *Plasmodium falciparum*

1.2.1 Taxonomy and Characteristic Features

Malaria is caused by eukaryotic protozoan parasites of the *Plasmodium* genus which belongs to the order of Haemosporida whithin the phylum of Apicomplexa. Five species of *Plasmodium* are known to infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Figure 1.2). Overall, about 200 different species of *Plasmodium* have been characterized, infecting various vertebrate hosts including reptiles, birds and mammals (Keeling and Rayner 2015). *P. falciparum* stands out from the other four human pathogens due to the expression of a specialized surface antigen, *Pf*EMP1, which is linked to severe disease (Hviid and Jensen 2015) (see sections 1.1.2 and 1.2.3). This feature is shared by the *Plasmodium*-subgenus of *Laverania*, of which *P. falciparum* is the only member known to infect humans (Figure 1.2).

Apicomplexa are obligate intracellular parasites named after their apical complex that confers the ability to invade host cells. It consists of parasite-specific secretory organelles called micronemes and rhoptries, which release their content after the parasite's attachment to the host cell. Their secretion serves to establish a tight intracellular connection, to harness the power of the parasital actinomyosin motor for invasion and to direct the formation of the parasitophorous vacuole (PV) (Gubbels and Duraisingh 2012). Additional secretory organelles called dense granules are thought to play a role in host cell modification and PV-formation (Gubbels and Duraisingh 2012). A unifyling feature of the phylum is considered to be the ability of the invading stages (zoites) to actively enter the host cell, which can manifest itself in a phenomenon termed gliding motility when the zoite is placed on a suitable substrate (Heintzelman 2015).

Another characteristic Apicomplexan organelle is the apicoplast, a plastid derived from a secondary endosymbiosis event when the ancestor of Apicomplexa incorporated a red algae. Over the course of evolution, it lost most metabolic functions, including photosynthesis but remains essential in *Plasmodium* parasites for isoprenoid synthesis (Yeh and DeRisi 2011; McFadden and Yeh 2017).

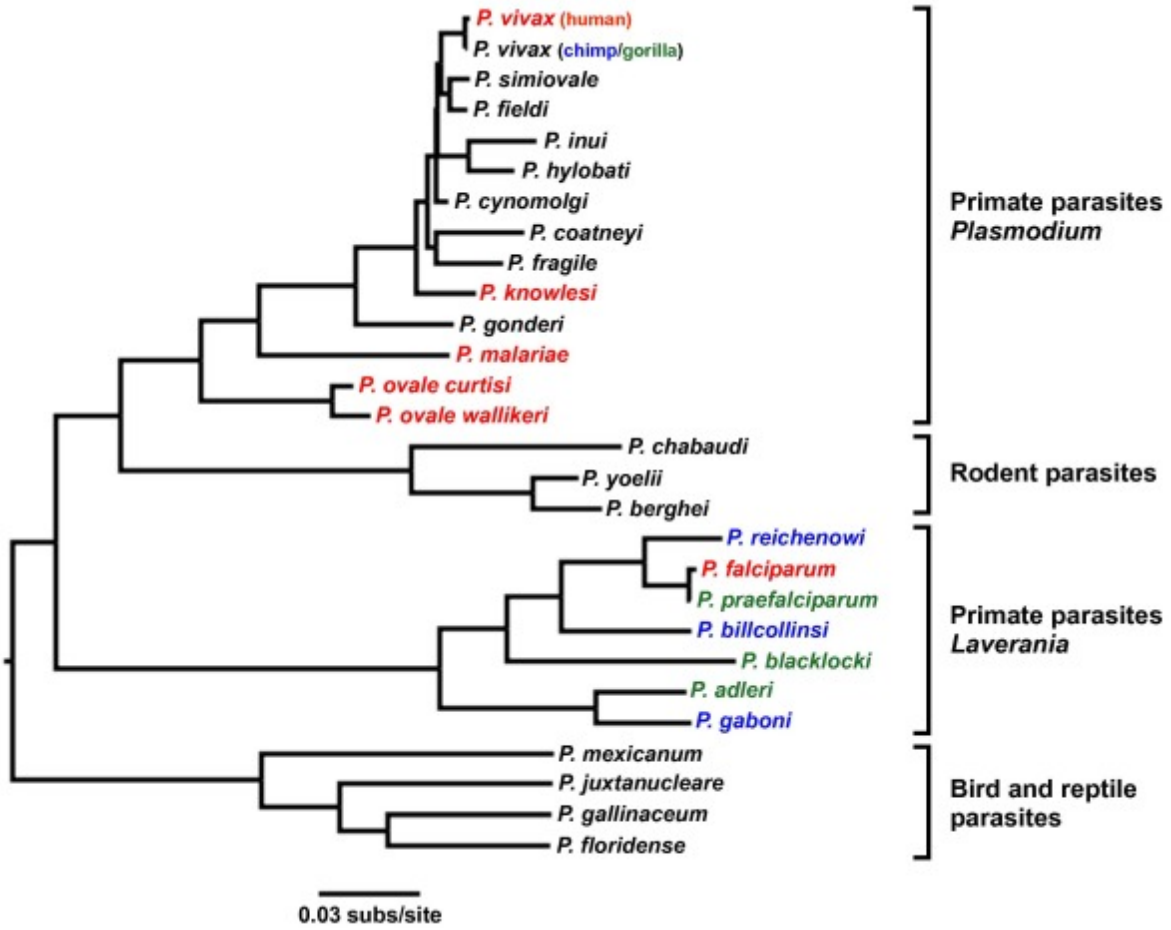

Figure (1.2): Phylogenetic tree of *Laverania* within exemplary members of the *Plasmodium* genus. Labels are colored for different hosts: red - human; blue - chimpanzee; green - gorilla . Graphic modified from (Loy et al. 2017).

Unique to plasmodial blood stages is the formation of a lysosome-like organelle, termed food vacuole or digestive vacuole, that becomes apparent in late ring stages and is discarded at the moment of schizont rupture (see section 1.2.2). During the intracellular growth of the parasite, hemoglobin is taken up from the host erythrocyte and catabolized in the food vacuole (Sigala and Goldberg 2014). Thus liberated heme is detoxified and stored in biocrystals called hemozoin (Olivier et al. 2014). Although it plays an important role in malaria pathophysiology as well as in the mode of action of antimalarials, the formation of this compartment and the mechanism of hemoglobin uptake remains poorly understood (Francis, Sullivan, and Goldberg 1997; Sigala and Goldberg 2014). Currently, competing models of food vacuole formation and hemoglobin uptake exist (Lazarus, Schneider, and Taraschi 2008; Elliott et al. 2008; Abu Bakar et al. 2010).

1.2.2 Life Cycle

P. falciparum undergoes a haplontic life cycle that involves infection of both humans and female mosquitos of the species *Anopheles* (Figure 1.3).

The saliva of an infected mosquito contains a haploid, infectious form of the parasite called sporozoites. With a bite of this mosquito, sporozoites enter the human blood stream and reach the liver, where they develop in hepatocytes. Within the host cell the parasite resides in the PV where it undergoes multiple rounds of mitosis and matures into a liver-schizont containing up to 30.000 merozoites, the invasive form of *Plasmodium* blood stages (Cowman et al. 2016).

Upon the rupture of a mature liver-schizont, merozoites are released in membraneous sacs termed merosomes (Sturm et al. 2006). In the blood stream, the merozoites are discharged from the merosomes and invade erythrocytes. Again, with its entry into the host cell, the parasite immediately forms a PV wherein it resides during its intraerythrocytic development (Cowman et al. 2017; Alaganan, Singh, and Chitnis 2017).

During its blood stage, *P. falciparum* undergoes cyclic rounds of replication, infecting new RBCs in every cycle. Over the course of 48 hours, the parasite grows inside the RBC from the so-called ring stage into the trophozoite and finally the schizont stage. This schizont stage is marked by mitotic replication and the formation of up to 32 new merozoites. The cycle is completed with the rupture of the host cell, the release of the newly formed merozoites into the blood stream and the invasion of new RBCs (Figure 1.3) (Spillman, Beck, and Goldberg 2015; Cowman et al. 2016).

Once a merozoite has successfully invaded an erythrocyte, it quickly implements a plethora of molecular adjustments in its host cell environment to create optimal conditions for its asexual replication. This includes an increase of the RBC membrane permeability to facilitate nutrient uptake. Other host cell modifications lead to alterations in the erythrocyte rigidity and the presentation of surface antigens, both in order to evade clearance of infected RBCs (iRBCs) in the spleen and by the immune system (see section 1.2.3) (Buffet et al. 2011; Boddey and Cowman 2013; Spillman, Beck, and Goldberg 2015; Cowman et al. 2016).

A small fraction of newly invading parasites differentiate into gametocytes (Figure 1.3). If these haploid sexual precursor forms are ingested by a female *Anopheles* mosquito during a blood meal, they form gametes in the insect's midgut. Fertilization results in a diploid zygote that develops into an ookinete. The ookinete undergoes meiosis and actively migrates to the space between the mosquito's midgut epithelium and the luminal side of its basement membrane (Angrisano et al. 2012). Here, it transforms into an oocyst, forming several hundreds of sporozoites which are released after 10-14 days and migrate to the salivary glands, from where they can be transmitted to a new human host (Cowman et al. 2016).

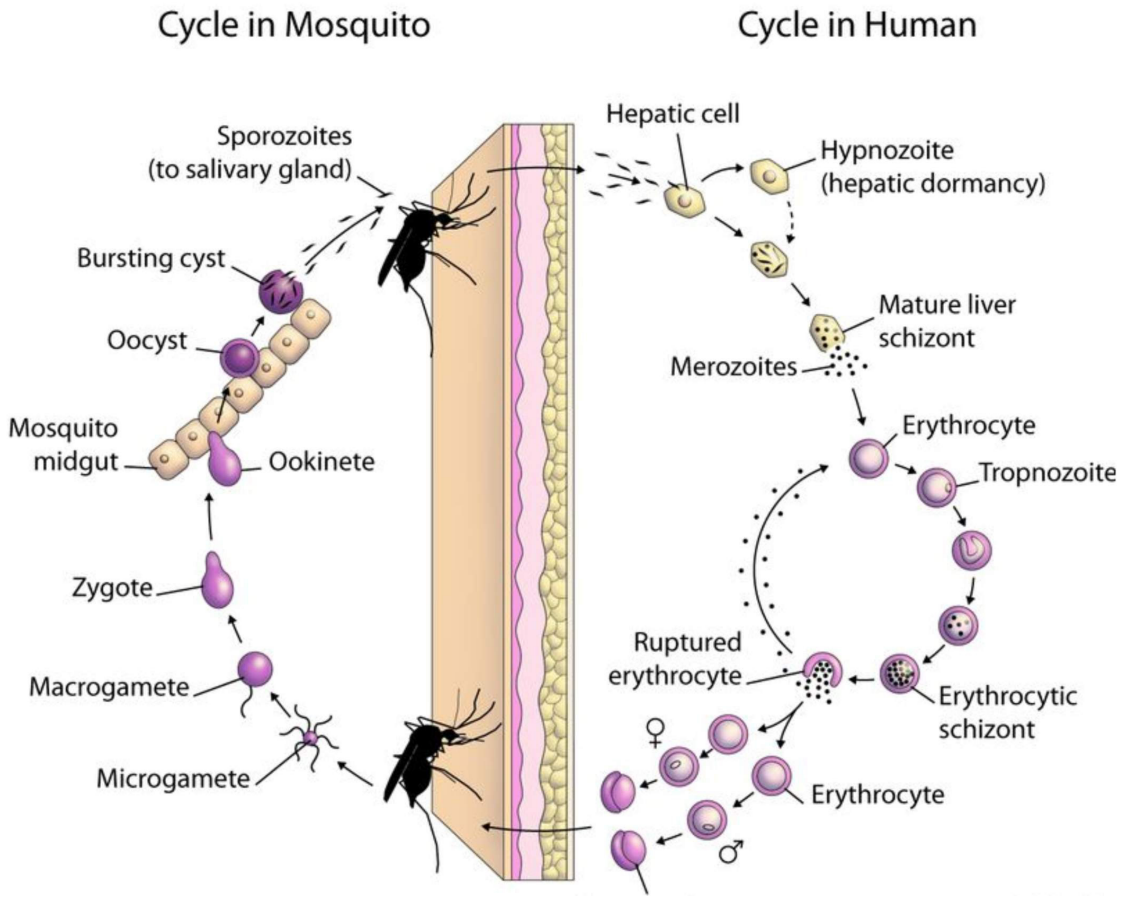

Figure (1.3): The Life cycle of Malaria parasites. Source: "Life cycle of the malaria parasite" from Epidemiology of Infectious Diseases. Available at: `http://ocw.jhsph.edu`. Copyright © Johns Hopkins Bloomberg School of Public Health. Creative Commons BY-NC-SA.

1.2.3 Features of the Infected Erythrocyte

The symptoms of malaria are caused by the blood stage of the pathogen. While fever and other general signs of inflammation are triggered by the exposure of host cell debris and parasite antigens to the immune system, more severe symptoms are linked to modifications of the iRBC by *P. falciparum* (see sections 1.1.2 and 1.2.2). These host cell modifications do not only play a pivotal role in the pathogenesis of *falciparum*-malaria, but are also essential for the parasite's propagation during its blood stage (Spillman, Beck, and Goldberg 2015; Cowman et al. 2016).

The iRBC's Exomembrane System

Within the infected erythrocyte, the parasite resides in the PV (see section 1.2.2). Thus, in order to reach the iRBC's cytosol, exported proteins must cross the parasite PPM, the PV lumen and the PVM.

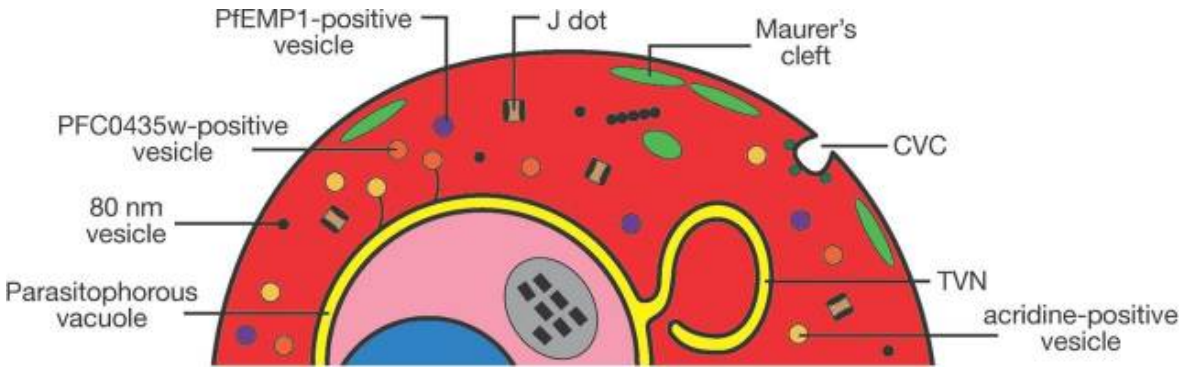

Figure (1.4): Scheme of parasite-induced membraneous structures in the iRBC. The exomembrane system comprises the PVM with the TVN, Maurer's Clefts (MC), and different types of vesicles. Pink: parasite cytosol. J dots: chaperonin complex containing J-domain proteins. CVC: caveola-vesicle complex, a structure induced by non-*falciparum* Plasmodia. Source: (Sherling and Ooij 2016).

Parasite antigens such as *Pf*EMP1 that are exposed on the iRBC's exterior surface require further trafficking to reach their destination. While many questions remain concerning the transport pathways of surface antigens, a variety of membranous structures induced by the parasite have been identified, some of which may serve as intermediate stations for cargo destined for the iRBC surface. The entirety of these structures is subsumed under the term exomembrane system and comprises the PVM and the tubulovesicular network (TVN) which is thought to be formed by PVM protrusions, membraneous structures called Maurer's Clefts (MC), and smaller vesicles and mobile compartments that have only begun to be characterized (Figure 1.4) (Hanssen et al. 2010; Mundwiler-Pachlatko and Beck 2013; Sherling and Ooij 2016). A number of exported proteins have been identified as markers of these extraparasital structures and have been implied in their formation and differentiation (Sherling and Ooij 2016).

Surface Antigens

During its blood stage, *P. falciparum* displays a variety of antigens on the infected erythrocyte surface. These proteins are translocated across the PVM and trafficked through the host cell to reach their final destination. Surface antigens are members of multigene families and

undergo antigenic variation in order to evade the host immune system. *Pf*EMP1, the major virulence factor of *falciparum*-malaria, is encoded by the *var*-gene family (about 60 genes per parasite) that appears to be unique to *Laverania*, and certain variants of which are associated with severe forms of malaria (Hviid and Jensen 2015). Other gene families coding for surface antigens are the repetitive interspersed family (*rif*) and sub-telomeric variable open reading frame (*Stevor*) (Smith et al. 2013; Chan, Fowkes, and Beeson 2014).

Cytoskeleton reinforcement

Functionally linked to exported surface antigens are proteins interacting with the host cell cytoskeleton. *Pf*EMP1 is anchored below the erythrocyte membrane in electron-dense knobs, with their main component being knob-associated histidine-rich protein (KAHRP) (Wickham et al. 2001; Rug et al. 2006). Several other exported proteins have been linked to an increase in the iRBC's rigidity that contributes to the sequestration of mature parasite stages in different organs (Maier et al. 2009; Spillman, Beck, and Goldberg 2015).

Alterations of RBC membrane permeability

It has been established that the membrane of iRBCs has an increased permeability for ions and many small molecules such as sugars, amino acids and other nutrients. The molecular mechanism conferring these altered properties has not been identified with certainty. Clag3 (cytoadherence-linked asexual protein 3) is a rhoptry protein that is thought to be inserted into the iRBC membrane during invasion and has been implied in the formation of a "*Plasmodium* surface anion channel" (PSAC). However, Clag3 alone seems to be insufficient to establish a fully functional PSAC (Beck et al. 2014). Recently, two further rhoptry proteins termed RhopH2 and RhopH3 which are known to form complexes with Clag3 have been linked to PSAC (Ito, Schureck, and Desai 2017; Sherling et al. 2017; Counihan et al. 2017). Further research will be needed to elucidate the mechanism by which these proteins contribute to the transport of nutrients and metabolic remains across the RBC membrane.

1.3 Protein Export During the *Plasmodium falciparum* Blood Stage

Proteins exported into the host cell by the parasite during its blood stage are essential for its intracellular development and contribute decisively to the pathogenesis of malaria, as outlined in the previous sections. In order to be exported, a newly synthesized protein must be trafficked to and across the PPM, the PV and finally the PVM before it reaches the host cell (Figure 1.5).

The first stage of this route is considered to be equivalent to the default secretory pathway of eukaryotes (Cleves and Bankaitis 1992) in which proteins are trafficked by vesicles from the endoplasmic reticulum (ER) via the Golgi towards the PPM (Deponte et al. 2012). After fusion of a transport vesicle with the PPM, soluble proteins are released into the PV, while transmembrane (TM) proteins would end up spanning the PPM. It is not known how exported TM proteins are extracted from the PPM to continue their pathway. While there are several conceivable models for this process (see section 4.1.5), it has also been suggested that such proteins could be synthesized and trafficked towards the PV in a soluble state (Deponte et al. 2012). Finally, exported proteins are thought to be translocated into the host cell by the PTEX-complex (Crabb, Koning-Ward, and Gilson 2010) which is located at the luminal side of the PVM (see section 1.3.2).

Most known exported proteins in *P. falciparum* parasites possess an N-terminal signal peptide (SP) recruiting the polypeptide to the ER during translation, followed by a conserved motif of five amino acids, called PEXEL (plasmodial export element), that acts as an export signal (Marti et al. 2004; Hiller et al. 2004). Thus, there is a "typical" structure for the N-terminal region of exported proteins, and about 400 genes are predicted to code for PEXEL-containing exported proteins (Sargeant et al. 2006). However, a substantial number of exported proteins deviate from this structure (Heiber et al. 2013; Spielmann and Gilberger 2010; Schulze et al. 2015) (see section 1.3.1).

1.3.1 Structure and Trafficking of Exported Proteins

PEXEL Proteins

PEXEL-proteins include soluble proteins, peripheral and integral membrane proteins. They are targeted to various locations inside the host cell and to its surface, contributing in several

critical ways to the pathogen's virulence (Spillman, Beck, and Goldberg 2015).

The SP preceding the PEXEL motif is often recessed from the N-terminus by up to 80 amino acids, which is an unusually large distance compared to other secreted proteins (Lingelbach 1993; Nacer et al. 2001). However, this elongated N-terminus is not needed to target a protein to the ER or for export and its function to date is unknown (Meyer et al. 2017).

The pentameric PEXEL motif (Hiller et al. 2004; Marti et al. 2004) is found downstream after a classical hydrophobic N-terminal signal peptide and has the consensus `R.L.[DEQ]` (with `R` for arginine, `L` for leucine, `[DEQ]` for any one of aspartic acid `(D)`, glutamic acid `(E)` or glutamine `(Q)`, and the dots signifying an uncharged amino acid).

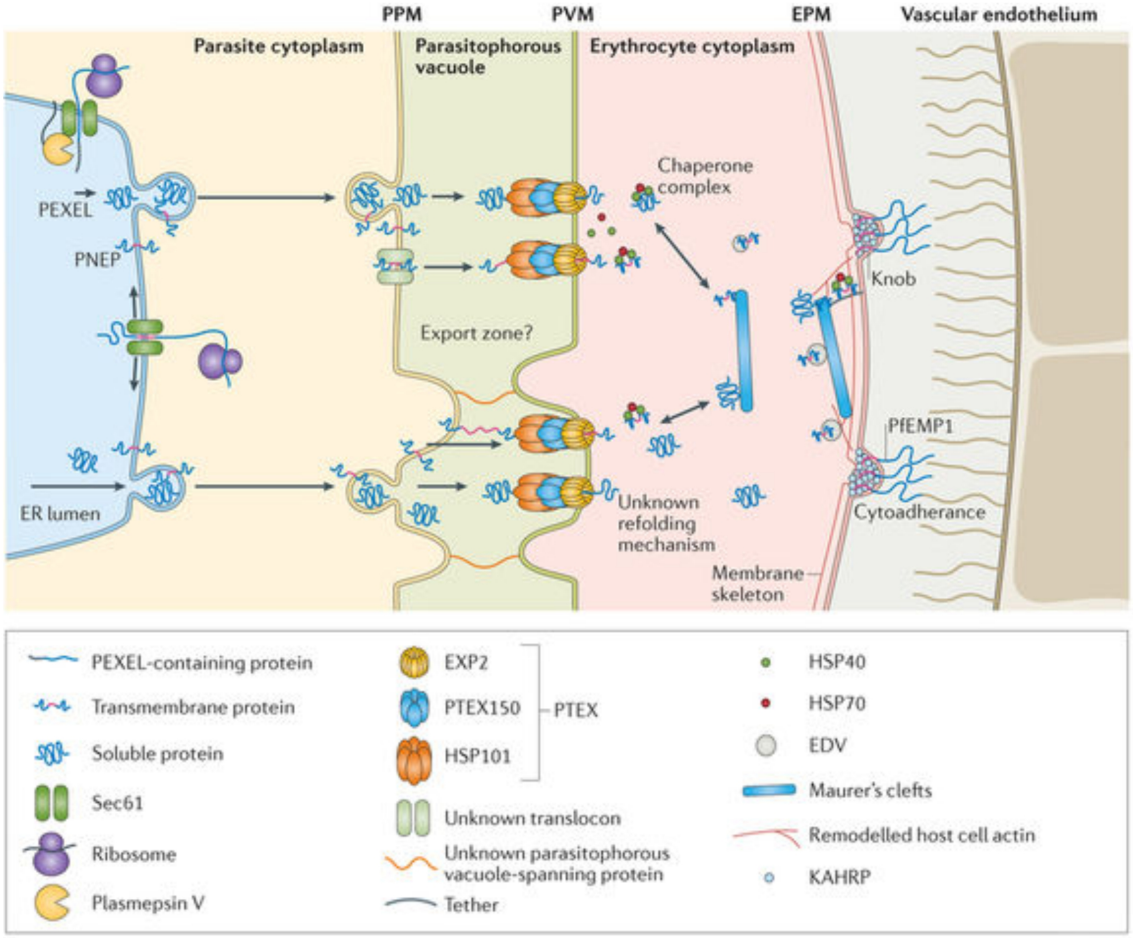

Figure (1.5): Scheme of the trafficking of parasite proteins exported into the host cell. Graphic modified from (Koning-Ward et al. 2016).

PEXEL Processing

The PEXEL-motif downstream of the signal peptide is cleaved by the ER-resident aspartic protease Plasmepsin V (PMV). Cleavage occurs between residues 3 and 4 of the sequence, exposing an N-terminus of `.[DEQ]` which is subsequently acetylated (Chang et al. 2008; Boddey et al. 2009; Boddey et al. 2010; Russo et al. 2010). PMV-cleavage of the PEXEL-motif has been shown to be essential for the export of PEXEL-proteins and for parasite growth (Russo et al. 2010; Boddey et al. 2010; Boddey et al. 2013; Sleebs et al. 2014).

The conserved `R` and `L` residues of the PEXEL have been shown to be necessary for recognition and processing by PMV. Experimental constructs with mutations in these positions retained their PEXEL, were processed by the ER signal peptide peptidase and secreted into the PV. PEXEL-proteins with a mutated last residue (`D`, `E` or `Q`) are processed by PMV and also secreted into the PV but not beyond (Boddey et al. 2009; Spillman, Beck, and Goldberg 2015). This indicates that the "mature" processed PEXEL N-terminus is required to mediate export. N-terminal acetylation seems to be common in ER-processed proteins and its importance for export is unclear (Boddey et al. 2009; Chang et al. 2008; Spillman, Beck, and Goldberg 2015).

The fact that PMV is essential for the export of PEXEL-proteins led to the question whether the exposure of a "mature" `.[EDQ]` N-terminus is sufficient for export or if PEXEL-cleavage by PMV accounts for some additional factor that mediates recognition by the export machinery. Such an additional factor could be the handover of the substrate to auxiliary proteins such as chaperones or some other as yet unrecognized substrate modification. This question was addressed in several studies by creating reporter constructs with N-termini mimicking a processed PEXEL. Some of those constructs were not exported (Gruring et al. 2012; Boddey et al. 2010) while others were (Gruring et al. 2012; Tarr et al. 2013). Taken together, these studies suggest that some N-termini may indeed allow export substrates to bypass PMV-cleavage depending on sequence information in addition to the "mature PEXEL" (Gruring et al. 2012; Tarr et al. 2013).

It has been shown that there exist proteins with motifs resembling the canonical PEXEL but with atypical residues in some positions of the pentamer that are processed and exported like PEXEL proteins. Whether these "non-canonical PEXELs" are functional appears to depend on further sequence information downstream of the motif (Schulze et al. 2015).

Several studies have implied PI(3)P-binding in the export of PEXEL-proteins (Bhattacharjee et al. 2012). However, a recent attempt to reproduce these results failed (Boddey et al. 2016). As the presence of PI(3)P in the ER lumen is also not commonly accepted, further work is needed to substantiate a role of PI(3)P-binding in protein export.

PEXEL-negative exported proteins

Several parasite proteins that are exported into the host cell despite lacking a PEXEL have been identified. This class of proteins has therefore been termed PEXEL-negative exported proteins (PNEPs) (Spielmann and Gilberger 2010; Spielmann and Gilberger 2015). PNEPs vary greatly in their structure, including both soluble proteins and integral TM proteins either with or without an ER-type SP (Heiber et al. 2013; Spielmann and Gilberger 2015; Spillman, Beck, and Goldberg 2015). Regardless of whether they possess an ER-type signal peptide or not, PNEPs are thought to follow the default secretory pathway, as their export is inhibited by Brefeldin A which abrogates ER-to-Golgi trafficking (Saridaki et al. 2009; Haase et al. 2009). In PNEPs lacking a signal peptide, a TM-domain directs the substrate into the secretory pathway and mediates export (Haase et al. 2009; Deponte et al. 2012). Replacing the TM-domain of a PNEP with that of a non-exported protein still lets the resulting construct enter the secretory pathway but abrogates its transport beyond the PVM (Saridaki et al. 2009; Haase et al. 2009; Gruring et al. 2012).

Truncation studies of different PNEPs showed that their N-termini, among other features, play a role in host cell targeting. Replacing PNEP-N-termini with "mature" PEXEL-N-termini does not interfere with export. It is thus assumed that sequence information necessary for protein export is found in these N-termini (Gruring et al. 2012; Ullrich 2016). However, it remains elusive how exactly the composition of PNEP N-termini, TM domains (TMD) and their adjacent regions facilitate export (Heiber et al. 2013; Gruring et al. 2012; Ullrich 2016; Blancke-Soares 2016).

1.3.2 The PTEX Complex

A combination of proteomic studies of PVM-lipid rafts, *in-silico* analysis and pull-down assays identified PTEX a complex of initially five components that appears to function as a translocon for exported proteins at the PPM-PVM interface (Koning-Ward et al. 2009). This complex comprises a putative "core" consisting of the three proteins HSP101 (heat shock protein 101), PTEX150 and EXP2 (exported protein 2) and the "auxiliary" proteins PTEX88 and TRX2 (thioredoxin 2) (Koning-Ward et al. 2009; Bullen et al. 2012; Matz, Matuschewski, and Kooij 2013) (Figure 1.6). Recently, several more proteins that seem to associate with PTEX-components have been identified: PV1, PV2, EXP3, Pf113 and HSP70-x (Elsworth et al. 2016; Mesen-Ramirez et al. 2016; Batinovic et al. 2017).

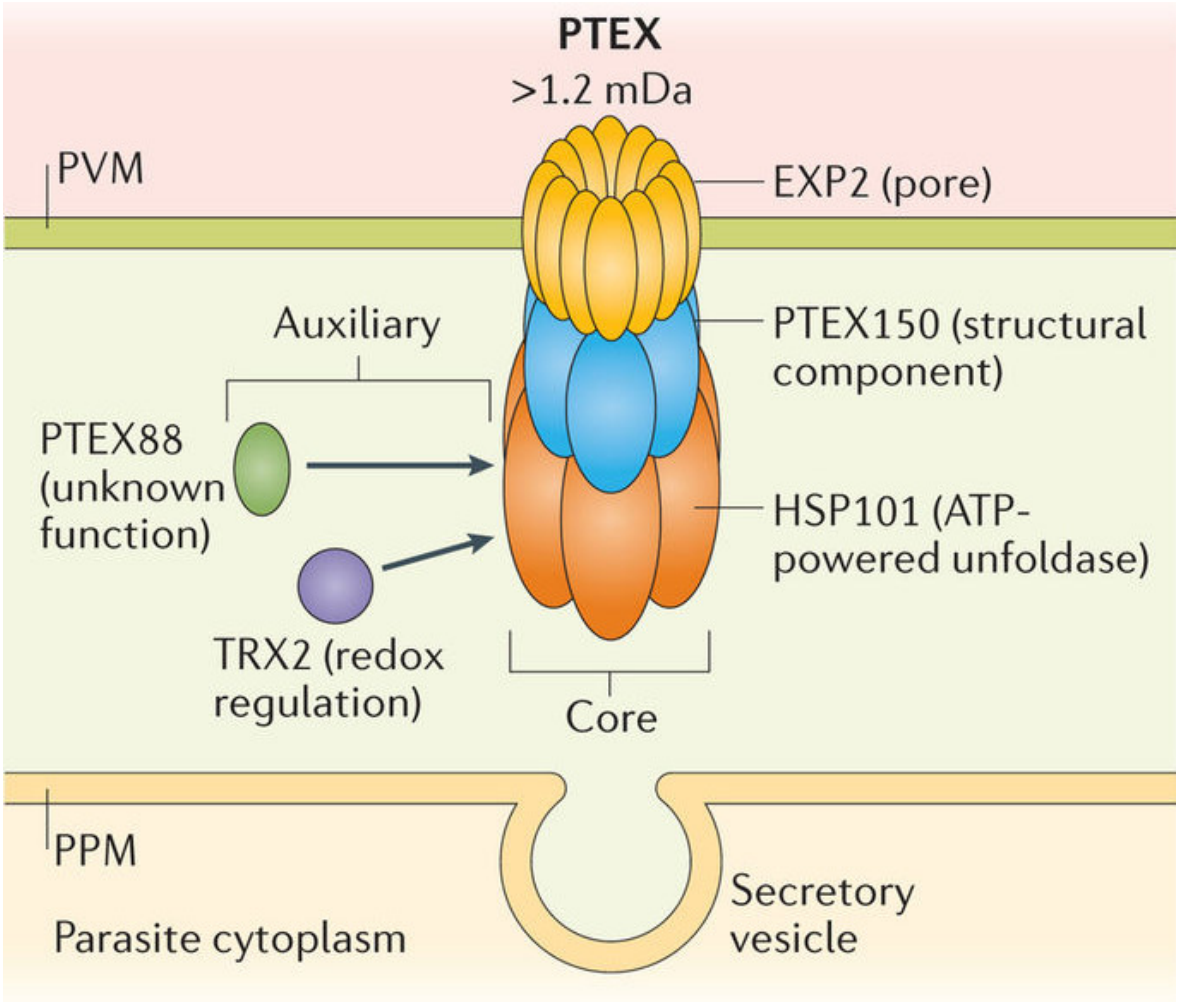

Figure (1.6): Hypothetical organisation of PTEX components. The membrane-spanning pore of the translocon is supposed to be formed by homooligomeric EXP2, while PTEX150 and HSP101 assemble in hexamers on the luminal side of the PVM. The auxiliary components PTEX88 and Trx2 associate with the core components in the PV lumen. Source: (Koning-Ward et al. 2016).

HSP101

HSP101 is a HSP100/Clp (caseinolytic protease) A/B-type chaperone and AAA+ ATPase (Koning-Ward et al. 2009). Members of the HSP100-family are involved in diverse cellular processes including protein disaggregation, degradation and translocation and play a role in protein import into plasmids (Schirmer et al. 1996; Trosch et al. 2015). Binding of ATP or ADP enables the formation of circular HSP100-hexamers with a narrow central channel through which a substrate can be unfolded and translocated (Schirmer et al. 1996). The diameter of this pore has been shown to be between 10 and 30 Å in different members of the HSP100 family with a length of 80 Å in *S.cerevisiae*-HSP104 (Schirmer et al. 1996;

Zolkiewski 2006; Gates et al. 2017). The energy for substrate propulsion is generated by ATP-hydrolysis (Schirmer et al. 1996).

Orthologs of PfHSP101 are found exclusively within the *Plasmodium* genus (Bullen et al. 2012). During the erythrocytic cycle, HSP101 biosynthesis peaks through the schizont and early ring stages (Bullen et al. 2012). In merozoites, the protein localizes to dense granules (Bullen et al. 2012). PTEX150 and EXP2 have been shown to colocalize with HSP101 in dense granules, which would allow PTEX to assemble in the PV directly after invasion (Koning-Ward et al. 2009; Bullen et al. 2012).

During the intraerythrocytic stages, HSP101 localizes to distinct foci in the parasite periphery, likely corresponding to the inner face of the PVM (Koning-Ward et al. 2009). It has been shown that upon destabilisation (Beck et al. 2014) or knockdown (Elsworth et al. 2014) of HSP101, proteins that are normally exported into the RBC cytosol accumulate in the parasite periphery and parasites fail to develop into schizonts. These findings indicate that HSP101 as a core component of PTEX is required for protein translocation into the host cell and that protein export is essential for parasite development.

Interestingly, HSP101 is not detected during *P.berghei* liver stages and fluorescence-tagged PEXEL-reporters were not exported in hepatocytes (Kalanon et al. 2016). The role of PTEX for the development of the parasite in this stage is yet to be determined.

EXP2

Although EXP2 lacks a predicted TM domain, it is considered to be the component of PTEX most likely to form the membrane pore of the translocon in the PVM. It appears to be tightly membrane-associated and to bear some structural similarity to the pore-forming *E.coli* haemolysin E (Sanders et al. 2007; Koning-Ward et al. 2009). GRA17 (dense granule protein 17), an EXP2 homolog in *Toxoplasma gondii* which is tightly PVM-associated, mediates the exchange of small molecules across the PVM in this parasite, and its knockout-phenotype can be rescued by *Pf*EXP2 (Gold et al. 2015). These findings indicate that EXP2 might be involved in the uptake of solutes by the parasite in addition to its role protein export. *In-vitro*, recombinant EXP2 was reported to form oligomeric pores of 34 Å diameter and to be able to lyse erythrocytes similar to hemolysins, although the significance of these findings for its *in-vivo* function will need verification (Hakamada et al. 2017).

EXP2 synthesis during the blood cycle peaks in the trophozoite stage (Bullen et al. 2012). It co-localizes with HSP101 and PTEX150 in the apical dense granules in merozoites and at the PV-PVM interface in trophozoites and schizonts (Koning-Ward et al. 2009; Bullen et al. 2012).

EXP2 has been shown to contribute to the intrahepatic development and transition to the

blood stage of *P. berghei* parasites, although PTEX-mediated export seems to be absent in liver stages (Kalanon et al. 2016).

PTEX150

To date, PTEX150 lacks any known homologs outside of the *Plasmodium* genus and is devoid of motifs that could indicate its biological function (Elsworth et al. 2016; Koning-Ward et al. 2009). However, knockdown of the gene blocked protein export in blood stages and prevented parasite maturation into schizonts (Elsworth et al. 2014). This protein therefore is essential for protein export and a core component of the PTEX complex.

It was suggested that PTEX150 may play a role in stabilising the PTEX machinery, as it could be shown to consistently associate with EXP2 and HSP101 (Koning-Ward et al. 2009; Bullen et al. 2012; Mesen-Ramirez et al. 2016; Elsworth et al. 2016). In contrast, EXP2 and HSP101 both co-precipitate less with each other than with PTEX150, suggesting that PTEX150 may link the two other core components together (Bullen et al. 2012).

PTEX88

PTEX88 also lacks known homologs outside of the *Plasmodium* genus and does not contain regions suggestive of its biological function. PTEX88-deficient *P. berghei* parasites showed unimpaired protein export during blood stages but markedly reduced virulence *in-vivo* (Matz, Matuschewski, and Kooij 2013) as they failed to induce cerebral malaria and showed reduced sequestration to peripheral tissue as well as accelerated elimination in the spleen (Matz et al. 2015a; Chisholm et al. 2016). Conditional knockout of PTEX88 in *P. falciparum* reduced the binding of iRBCs to the endothelial receptor CD36. Thus, it has been hypothesized that this PTEX component only regulates the export of very specific, unknown substrates, or that its impact on PTEX function is too subtle to be visualized by fluorescence-tagged reporters (Chisholm et al. 2016).

TRX2

The thioredoxin-like TRX2 has been suggested to serve auxiliary functions for protein export such as substrate unfolding or regulation of the PTEX core (Koning-Ward et al. 2009). The gene was successfully disrupted in *P.berghei* and led to a phenotype that showed retained virulence but a slowed progress through the intraerythrocytic life cycle (Matz, Matuschewski, and Kooij 2013; Matthews et al. 2013). Strikingly, to this day, the localisation of TRX2 to the

PV could not unequivocally be confirmed as it frequently shows strong foci in the parasite rather than the expected peripheral location (Matz, Matuschewski, and Kooij 2013).

Newly determined proteins associated with PTEX

In Blue Native gels the core PTEX-complex migrates in a >1230 kDa band (Bullen et al. 2012; Elsworth et al. 2016). While MS-analysis failed to detect TRX2 in this band, it yielded not only PTEX88 but also three additional proteins that were formerly not associated to the complex, namely PV1, Pf113, and HSP70-x (Elsworth et al. 2016). These three proteins where independently co-precipitated with HA-tagged EXP2 (Mesen-Ramirez et al. 2016).

PV1 has no homologs outside the *Plasmodium* genus and localises to the PV lumen (Chu, Lingelbach, and Przyborski 2011). A recent study showed that it is dispensable for *in-vitro growth*, but linked it, together with two other PV-proteins, PV2 and EXP3, to the export of *Pf*EMP1. The complex of those three proteins was termed "EPIC" (Exported Protein-Interacting Complex). The same study confirmed that PV1 interacts with HSP70-x (Batinovic et al. 2017). Furthermore, it was shown that PV1 interacts with exported proteins in the PV (Morita et al. 2018).

*Pf*113 is a GPI(-glycosylphosphatidylinositol)-anchored protein that localises to the merozoite surface (Sanders et al. 2005; Sanders et al. 2007) where it serves as an anchor for the erythrocyte receptor ligand RH5 (Galaway et al. 2017). One study has linked *Pf*113 to the conversion from sporozoite to liver stage (Offeddu et al. 2014), while it was also found by two independent studies to co-precipitate with PTEX components (Elsworth et al. 2016; Mesen-Ramirez et al. 2016).

HSP70-x is a member of the HSP70-chaperonin family that has been linked to protein import in chloroplasts among many other functions such as the regulation of protein interactions (Trosch et al. 2015). Fluorescence-tagging and cell fractionation assays suggest that it is partially retained in the PV and partially exported into the RBC cytosol where it is associated with highly mobile foci called J-dots (Kulzer et al. 2010; Kulzer et al. 2012). Immunofluorescence assays suggested partial colocalisation with *Pf*EMP1 or MCs, respectively (Kulzer et al. 2012; Grover et al. 2013), but its exact location remains to be clarified. In J-dots HSP70-x colocalises with PEXEL-containing exported members of the HSP40-family (Kulzer et al. 2012). HSP40s often act as co-factors of HSP70s to modulate protein interactions (Pesce and Blatch 2014). HSP70-x is of particular interest as a PTEX-associated protein because members of the HSP70 family are known to provide energy for protein translocation processes (Matouschek, Pfanner, and Voos 2000). However, a recent study reported a successful knockout of the gene coding for the chaperone. This HSP70-x deficient strain did not show any growth phenotype but rather a hampered display of *Pf*EMP1 on the erythrocyte surface,

suggesting a more subordinate role of HSP70-x, if any, in the export process (Charnaud et al. 2017).

1.3.3 Methods to Study Translocation Processes

To be translocated through a proteinaceous channel, polypeptides usually need to be unfolded (Eilers and Schatz 1986). In this study, we used two different systems to conditionally inhibit the unfolding of exported proteins.

Murine dihydrofolate reductase (mDHFR) has previously been used as a means to study the role of the folding state of a protein substrate for translocation processes in various experimental setups, including live *P. falciparum* parasites (Eilers and Schatz 1986; Gehde et al. 2009; Heiber et al. 2013; Gruring et al. 2012). Fusing translocation substrates to a mDHFR-domain allows conditional inhibition of those substrates' unfolding by the addition of a folate analogue that binds and stabilizes the mDHFR-domain (Eilers and Schatz 1986). Using this system, it was shown that reporter constructs derived from PEXEL-N-termini, integral membrane-PNEPs and soluble PNEPs fused to mDHFR undergo a translocation step at the host-pathogen-interface in order to reach the host cell, as they accumulate in the parasite periphery upon the addition of WR99210 (WR), a folate analogue (Gehde et al. 2009; Gruring et al. 2012; Heiber et al. 2013).

A different approach to study translocation processes recently adapted for *P. falciparum* in our lab is the utilisation of bovine pancreatic trypsin inhibitor (BPTI) as a foldable domain (Mesen-Ramirez et al. 2016; Mesen-Ramirez 2016). BPTI is irreversibly stabilised in a folded state by three intramolecular disulfide-bridges that form under oxidising conditions (Vestweber and Schatz 1988). While the parasite cytosol is considered a reducing environment, the PV is thought to be oxidising (Kehr et al. 2010; Kasozi et al. 2013; Withers-Martinez et al. 2014), leading to the stabilisation of a BPTI-moiety once it is exposed to this compartment.

1.4 Aim of this Thesis

PTEX is thought to play a key role in the export of proteins during *P. falciparum* blood stages by translocating substrates across the PVM. It was previously shown that the trafficking of

exported proteins to the host cell requires an unfolding-dependent translocation step. In this study, we aimed to establish whether this is also the case for TM PEXEL proteins, a class of exported proteins that had not been previously investigated. In order to answer this question, we used constructs of exported proteins fused to mDHFR and green fluorescent protein (GFP, (Chalfie et al. 1994)).

Preliminary data suggested that, in contrast to previously studied mDHFR constructs, certain constructs are arrested during their translocation in a way that prevents the export of other proteins which use the same pore (Gehde et al. 2009; Gruring et al. 2012; Heiber et al. 2013; Mesen-Ramirez et al. 2016) (see section 3.2). The central aim of this thesis was to follow up on these findings by co-expressing different types of exported mDHFR constructs together with different types of exported mCherry-tagged proteins in order to test whether all types of exported proteins pass through the same translocon.

Lastly, it is unclear how exported TM proteins, which are thought to be trafficked to the PPM as integral membrane proteins, can become translocation substrates at the PVM. We expected that the analysis of the trafficking of different translocation substrates could help to shed light on the mechanism by which exported TM proteins are transported across the PPM.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Material	Manufacturer
Aceton	Merck, Darmstaft
Acrylamide/Bisacylamide-solution (40%)	Roth, Karlsruhe
Agar-Agar	Becton Dickinson, Heidelberg
Agarose	Invitrogen, Karlsruhe
AlbumaxII	Invitrogen, Karlsruhe
Ammoniumpersulfate (APS)	Merck, Darmstadt
Ampicillin	Roche, Mannheim
Blasticidin S (BSD)	Invitrogen, Karlsruhe
Bovine serum albumin (BSA)	Biomol, Hamburg
Desoxyribonucleotides (dNTPs)	Fermentas, St. Leon-Rot
4',6-Diamidino-2-Phenylindole (DAPI)	Roche, Mannheim
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Dulbecco's Phosphate Buffered Saline (DPBS)	Pan Biotech, Aidenbach
Ethanol (EtOH)	Merck, Darmstadt
Ethidiumbromide	Sigma-Aldrich, Steinheim
Ethylenediaminetetraacetate (EDTA)	Biomol, Hamburg
Giemsa Azure-Eosine-Methylblue Solution	Merck, Darmstadt

Isopropanol	Merck, Darmstadt
Methanol	Sigma-Aldrich, Steinheim
Milk powder	Roth, Karlsruhe
Percoll	BE Healthcare, Buckinghamshire, UK
Phenylmethylsulfonylfluoride (PMSF)	Sigma aldrich, Steinheim
Proteaseinhibitorcocktail ("Complete Mini")	Roche, Mannheim
RPMI (Roswell Park Memorial Institute) medium	Invitrogen, karlsruhe
Oligonucleotides	Invitrogen, Karlsruhe
Hydrochloric acid (HCl)	Merck, Darmstadt
Saponin	Sigma Aldrich, Steinheim
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, Steinheim
Sodium azide	Merck, Darmstadt
Sodium acetate	Merck, Darmstadt
Sorbitol	Sigma Aldrich, Steinheim
Tetramethylendiamine (TEMED)	Merck, Darmstadt
Tris(hydroxymethyl)aminomethane (TRIS)	Merck, Darmstadt
Triton X-100	Biomol, Hamburg
Water	Fresenius Kabi, Bad Homburg
WR99210 (WR)	Jacobus Pharmaceuticals, Maryland, USA

2.1.2 Ready-to-use Kits

Product	**Fabricant**
NucleoSpin® Plasmid Kit	Macherey-Nagel, Dueren
NucleSpin® Extract Kit	Macherey-Nagel, Dueren
QIAmp® DNA Mini Kit	Qiagen, Hilden
QIAGEN Plasmid Kit	Qiagen, Hilden
Western Blot "clarity" ECL-Detection Kit	Bio-Rad, Muenchen
Western Blot ECL-Detection Kit	Thermo Scientific, Schwerte

2.1.3 DNA- and Protein Standards

Product	Fabricant
GeneRuler 1kb DNA-Ladder	Thermo Scientific, Schwerte
PageRuler prestained protein-ladder	Thermo Scientific, Schwerte

2.1.4 Solutions, Buffers, Media

Solution	Contents
Microbiology and DNA precipitation	
10x LB Stock Solution	10% NaCl, 5% Peptone, 10% Yeast extract, in autoclaved dH_2O
1x LB fluid medium	1% NaCl, 0.5% Peptone, 1% Yeast extract in autoclaved dH_2O
LB + ampicillin fluid medium	Ampicillin added to 1x LB fluid medium at approx. 50°C to 100µg/ml
Ampicillin stock solution	Ampicillin 100 mg/ml in 70% EtOH
Solution for glycerine stabilates	50% Glycerine in 1x LB fluid medium
Sodiumacetate buffer for DNA precipitation	3 M Sodiumacetate pH 5.2
TRIS-EDTA buffer for DNA precipitation	10 mM TRIS-Hcl pH 8.0, 1mM EDTA, pH 8.0
Ribonucleic acid partitioning	
50x TAE buffer	2 M TRIS-base, 1 M pure acetic acid, 0.05 M EDTA, pH 8.0
Agarose gel	1% Agarose in 1x TAE buffer
6x Ribonucleic acid loading dye	40% Glycerine, 2.5% Xylencyanol, 2.5% Bromphenoleblue in dH_2O
Media and solutions for cell culture	

RPMI medium	1.587% RPMI 1640, 12 mM $NaHCO_3$, 6mM D-Glucose, 0.5% Albumax II, 0.2 mM Hypoxanthine, 0.4 mM Gentamycine, pH 7.2 in NaOH, filtered sterile
Malaria freezing solution (MFS)	4.2% D-Sorbitol, 0.9% NaCl, 28% Glycerine, filtered sterile
Malaria thawing olution (MTS)	3.5% NaCl in dH_2O, filtered sterile
Synchronizing solution	5% D-Sorbitol in sH_2O, filtered sterile
Transfection buffer (Cytomix)	120 mM KCl, 150 µmol $CaCl_2$, 2 mM EGTA, 5mM $MgCl_2$, 10 mM K_2HPO_4 / KH_2PO_4, pH 7.6, 25mM Hepes, pH 7.6, filtered sterile
Erythrocyte concentrates	sterile human 0+ concentrates (Blutbank, Universitätsklinikum Eppendorf, Hamburg)
10% Giemsa stain	10% Giemsa stain in dH_2O
WR99210 stock solution	20 mM in 1 ml DMSO, filtered sterile
WR99210 working solution	1:1000 stock solution in RPMI medium
Blasticidin S (BSD) working solution	5 mg/ml BSD in RPMI medium
Parasite lysis	
Saponin buffer 0.03%	0.03% in 1x PBS
Saponin buffer 0.015%	0.015% in 1x PBS
Lysis buffer	4% SDS, 0.5% Triton X-100, 0.5x PBS in dH_2O
Percoll gradient	
10x PBS	1.37 M NaCl, 26.8 mM KCl, 100 mM Na_2HPO_4 + $7H_2O$, 17.6 mM KH_2PO_4, in dH_2O, pH 7.4 with HCl, autoclaved
90% Percoll stock solution	90% (v/v) Percoll, 10% (v/v) 10x PBS
80% Percoll solution	89% (v/v) 90% Percoll stock solution, 11% RPMI medium, 4% (w/v) sorbitol, filtered sterile
60% Percoll solution	67% (v/v) 90% Percoll stock solution, 33% RPMI medium, 4% (w/v) sorbitol, filtered sterile

40% Percoll solution	44% (v/v) 90% Percoll stock solution, 56% RPMI medium, 4% (w/v) sorbitol, filtered sterile
Biochemical assays	
10x Running bufffer	250 mM TRIS-base, 1.92 M Glycine, 1% (w/v) SDS, in dH_2O
1x Running buffer	1:10 10x running buffer in dH_2O
Separation gel buffer	1.5 M TRIS-HCl pH 8.8
Separation gel (12%) for 10 ml	2.5 ml separation gel buffer, 4.25 ml H_2O, 3 ml 40% Acrylamide/Bisacrylamide solution, 100 µl 10% (w/v) SDS in dH_2O, 100 µl 10% (w/v) APS in dH_2O, 5 µl TEMED
Collecting gel buffer	0.5 M TRIS-HCl pH 6.8
Collecting gel (4%) for 5 ml	1.26 ml colecting gel buffer, 3.18 ml H_2O, 500 µl 40% Acrylamide/Bisacrylamide solution, 50 µl 10% (w/v) SDS in dH_2O, 50 µl 10% (w/v) APS in dH_2O, 5 µl TEMED
6x SDS sample buffer	375 mM TRIS-HCl pH 6.8, 12% (w/v) SDS, 60% (v/v) Glycerine, 0.6 M DTT, 0.06% (w/v) Bromphenoleblue
Western Blotting	
10x Western transfer buffer	250 mM TRIS-base, 1.92 M Glycine, 0.37% (w/v) SDS in dH_2O
1x Western transfer buffer	25 mM TRIS-base, 192 mM Glycine, 0.037% (w/v) SDS, 20% Methanol in dH_2O
Blocking solution	5% milk powder (w/v) in 1x PBS with 0.1% sodium acide
Wash buffer	1x PBS

2.1.5 Bacteria and Plasmodium Strains

Strain	Specification
Escherichia coli XL-10 Gold	ultracompetent *E.coli* strain, registered trademark of Stratagene California, La Jolla CA, USA
Plasmodium falciparum 3D7	strain obtained by limitind dilution of isolate NF54 (MRA-1000) (Walliker et al. 1987)

2.1.6 Enzymes

Product	Concentration	Manufacturer
FirePol DNA polymerase	[5 U/µl]	Bioline, Luckenwalde
Phusion HiFi DNA polymerase	[2 U/µl]	NEB, Ipswich, USA
T4 DNA ligase	[3 U/µl]	NEB, Ipswich, USA
AvrII RE	[4 U/µl]	NEB, Ipswich, USA
DpnI RE	[20 U/µl]	NEB, Ipswich, USA
KpnI HF RE	[20 U/µl]	NEB, Ipswich, USA
NheI HF RE	[20 U/µl]	NEB, Ipswich, USA
SmaI RE	[20 U/µl]	NEB, Ipswich, USA
SpeI RE	[10 U/µl]	NEB, Ipswich, USA
XbaI RE	[20 U/µl]	NEB, Ipswich, USA
XhoI RE	[20 U/µl]	NEB, Ipswich, USA
XmaI RE	[10 U/µl]	NEB, Ipswich, USA

2.1.7 Antibodies and Stains

Organism	Antibody	Dilution	Usage	Source
Mouse	α-GFP IgG	1:1000	Western Blot	Dianova, Hamburg
Rat	α-mCherry IgG	1:1000	Western Blot	Chromotek, Planegg-Martiensried
Goat	α-mouse HRP	1:3000	Western Blot	Dianova, Hamburg
Goat	α-rat HRP	1:3000	Western Blot	Dianova, Hamburg

2.1.8 Oligonucleotides

All listed oligonucleotides were synthesized by Sigma-Aldrich inc., Steinheim. Lyophilisates were dissolved in TRIS-EDTA (TE) buffer to a stock solution of 100 µmol, from which working solutions of 10 µmol were obtained at need by further dilution using TE buffer. Solutions were stored at -20°C.

Name	Sequence in 5'-3' direction
KAHRP KpnI fw	CTGTGGTACCATGAAAAGTTTTAAGAACAAAAATACTTTGAGG
KAHRP AvRII rv	TCCTCCTAGGACCACAGCATCCTCTTTTCTTCTTTTTCTTTCC
KAHRP XhoI fw	CTGTCTCGAGATGAAAAGTTTTAAGAACAAAAATACTTTGAGG
PTP1 fw XmaI	GTCTCCCGGGATGGTGAATAAAGATAATAGGAAAATTCATAAGGC
PTP1 rv AvrII	CAGTCCTAGGTTGGTTTTGTATATTTAAATTGTCATCTTGTTCC
REX3 KPN1 fw	CGGCGGTACCATGCAAACCCGTAAATATAATAAGATGTTG
REX3 AVR rv	TCCTCCTAGGTGAAGAACTTGTACTTGGTTTAGC
REX3 XhoI fw	CGGCCTCGAGATGCAAACCCGTAAATATAATAAGATGTTG
STEVOR 0631900 Kpn1 fw	GTCTGGTACCATGAAGATGTATTACCTTAAAATGTTATTG
STEVOR 0631900 Avr2	CAGTCCTAGGCTTACATAAATGTTTCTTGCATTCATGTTTCC
STEVOR 0900900 XhoI fw	GTCTCTCGAGATGAAGATGTATAACCTTAAAATGTTATTG

PTP1 fw XmaI	GTCTCCCGGGATGGTGAATAAAGATAATAGGAAAATTCATAAGGC
PTP1 rv AvrII	CAGTCCTAGGTTGGTTTTGTATATTTAAATTGTCATCTTGTTCC
PTP1 XhoI fw	CTGTCTCGAGATGGTGAATAAAGATAATAGGAAAATTCATAAGGC
BPTI Nhe1 fw	CTCGGCTAGCTCAACACCAGGTTGTGATACATCAAATCAAGCTAAAGC
BPTI-Kpn1-Stop-Xma1 rv	TCCTCCCGGGTTAGGTACCTAAATTTTCCCATGGACCTATAGCACC
PTP1 Spe1 rv	CAGTACTAGTTTGGTTTTGTATATTTAAATTGTCATCTTGTTCC

2.1.9 The pARL Vectors (Crabb et al. 2004a)

The plasmid pARL1a (-) constitutes a binary vector, i.e. a so-called "shuttle vector". This means it may be used to transform prokaryotes such as *E.coli* in order to generate sufficient amounts of DNA, as well as to transfect eukaryotes such as *P. falciparum* to express arbitrary engineered proteins. The cutting sites of the commercially available endonucleases AvrII and KpnI allow for the insert of any transgen which becomes consequently tagged by a C-terminal GFP domain. The GFP sequence in the pARL vector may be replaced by other tags such as mCherry (monomeric Cherry, red fluorescence) as done for this study. The sequence of the fusion protein is controlled by the promoter for the endogenous *P. falciparum*-crt ("Chloroquine resistance transporter") gene. This promotor is active during the whole asexual life cycle of the parasite and leads to a strong expression of the transgen. Engineered fusion proteins may thus be expressed in *P. falciparum* and analyzed microscopically and biochemically. To select for transgenic parasites, the gene for human dihydrofolate reductase (hDHFR) is used. This enzyme is not inhibited by the antifolate WR99210, as opposed to the endogenous DHFR of *P. falciparum*. DHFR, catalyzing the regeneration of tetrahydrofolate, is essential for the biosynthesis of purines and thus for cellular proliferation. Accordingly, transgenic parasites carrying the pARL1 vector are resistant to WR99210, while the replication of untransfected parasites is inhibited by the drug.

In the pARL2 vector, the hDHFR sequence was replaced by the gene for blasticidin deaminase (BSD), which confers resistance against the antibiotic blasticidin S. This drug inhibits the termination step of protein translation and consequently protein biosynthesis in prokaryotes as well as eukaryotes.

Double transgenic cell lines can be generated by subsequent transfections with both plasmids.

2.1.10 Sequencing

The sequencing in this study was carried out by SeqLab, Göttingen, via the desoxy-method according to Sanger. Following the firm protocol (`http://www.seqlab.de/index.php?=dna`), 600-700 ng of plasmid DNA were mixed with 20 pmol of sequencing oligonucleotides to a volume of 15 µl and mailed in for sequencing.

2.1.11 Disposables

Product	**Manufacturer**
Cover slips	Engelbrecht, Fürth
Disposable lab gloves, Latex	Kimberly Clark, Koblenz
Disposable lab gloves, Nitril	Kimberly Clark, Koblenz
Disposable Syringes	Braun, Melsungen
Disposable Needles	Braun, Melsungen
Object slides, glass	Roth, Karlsruhe
Glass beads 2.85 - 3.45 mm	Roth, Karlsruhe
Kryotubes	Sarstedt, Nürnberg
Leukosilk	BSN medical, Hamburg
Nitrocellulose transfer membrane	Schleicher & Schuell, Dasel
Parafilm	Pechiney, Mühltal
Pasteur Pipettes	Brand, Wertheim
Petri dishes (5 and 10 ml)	Sarstedt, Nürnberg
PCR tubes (0.2 ml)	Sarstedt, Nürnberg
Pipette tips (20, 200, 1000 µl)	Sarstedt, Nürnberg
Falcon tubes (15 and 50 ml)	Sarstedt, Nürnberg
Reaction tubes (1.5 and 2 ml)	Eppendorf, Hamburg
X-ray films	Agfa Healthcare, Mortsel, Belgium
Round filters	Schleicher & Schüll, Dassel
Serological Pipettes	Sarstedt, Nürnberg
Transfection cuvettes	Bio-Rad, München
Whatman filtering paper	Schleicher & Schüll, Dassel

2.1.12 Technical Devices

Device	Product name	Manufacturer
Acrylamide gel chamber	MiniProtean TetraCell	Bio-Rad, München
Agarose gel chamber	Sub Cell GT basic	Bio-Rad, München
Autoclav	V120	Systec, Wettenberg
Incubator, bacteria	CB53	Binder, Tuttlingen
Incubator, parasites	Heraeus B6200	Thermo Scientific, Scherte
Vacuum pump BVC Control	Vacuubrand, Deutschland	
Electroporator	X-Cell	Bio-Rad, München
Ice machine	AF-10	Scotsman, Vernon Hills, USA
Developer	Curix60	Agfa-Gvaert, Mortsel, Belgium
Analytical balance	Acculab Atilon-ATL	Sartorius, Göttingen
Fluorescence microscope	Axioskop 2 plus	Zeiss, Jena
Digital camera	C4742-95	Hamatsu Photonics, KK, Hamatsu, Japan
Confocal microscope	Olympus FV1000	Olympus, Hamburg
Cooling centrifuge	Heraeus Megafuge 1.0R	Thermo Scientific, Schwerte
Microwave	Micromaxx MM41568	Medion, Mühlheim
Magnetic stirrer	MR-Hei-Standard	Heidoplph, Schwabach
PCR mastercycler	epgradient	Eppendorf, Hamburg
Photometer	BioPhotometer plus	Eppendorf, Hamburg
Pipettes	Pipetman	Gilson, Middleton, USA
Ultrapure water unit	Milli-Q	Millipore, Bedford, USA
Roller mixer	SRT6	Bibby Scientifec, Staffordshire, USA
Power-Supply	PowerPac 300	Bio-Rad, München
Shaking incubator	MaxQ 4000	Thermo Scientific, Schwerte
Sterile hood	Sterilguard III, Advance	Baker company, Stanford, USA
Tabletop centrifuge	Centrifuge 5415 D	Eppendorf, Hamburg
Thermoblock	Thermomixer comfort	Eppendorf, Hamburg
Ultracentrifuge	Avanti J-26S XP	Beckmann-Coulter, Krefeld

Ultracentrifuge rotor	JA-12	Beckmann-Coulter, Krefeld
Ultracentrifuge rotor	JA-14	Beckmann-Coulter, Krefeld
Vortex	Genie 2	Scientific Industries, Bohemia, USA
Water bath	1004	GFL, Burgwedel

2.1.13 Databases and Online Resources

Name	**URL**	**Use**
ClustalOmega	`www.ebi.ac.uk/Tools/msa/clustalo/`	Sequence Alignment
NEBcutter	`tools.neb.com/NEBcutter2/`	Restriction Site Analysis
PlasmoDB	`www.plasmodb.org/plasmo/`	*P. falciparum* gene data base
PubMed	`www.ncbi.nlm.nih.gov/pubmed/`	References

2.1.14 Software

Product	**Use**
CorelDraw® 6	Image Editing
LaTeX	Document preparation system

2.2 Methods

2.2.1 General Methods

Sterilisation of materials and devices

Solutions, media, pipette tips and glass devices used in this study were autoclaved at 1.5 bar pressure and 121°C for at least 20 minutes. Heat sensitive substances were sterilized by sterile filtration at 0.2 um pore diameter.

2.2.2 Biomolecular and Microbiological Methods

Polymerase Chain Reaction (PCR) (Mullis and Faloona 1987; Saiki et al. 1988)

PCR is a method to amplify specific DNA fragments in vitro. A typical PCR requires a heat-resistant DNA polymerase, dNTPs (desoxyribonucleotide triphosphates), a DNA template, specific oligonucleotide primers for the desired fragment and suitable reaction buffer. A standard preparation is shown in table 2.13.

In this study, two different DNA polymerases were used. The Phusion ® High Fidelity DNA Polymerase has a "proof-reading" 3'-5'-exonuclease activity and therefore an error rate about two orders of magnitude lower than the benchmark Taq-Polymerase (Eckert and Kunkel 1990). It was used for all PCR reactions requiring high accuracy, i.e. to generate DNA for genetic engineering. The FIREPol ® DNA-polymerase lacks a "proof-reading" function and was used for colony screens of transformed *E.coli*.

The different steps of the PCR were cycled automatically in a programmable device. First, the DNA double strand was denaturated at 95°C. Second, the primers were allowed to bind at temperatures between 48° and 60°C, depending on their base composition and length (annealing). In the third step, the polymerisation (elongation) took place at temperatures between 65° and 72°C. Steps two and three were cycled 25-30 times. Reaction conditions were adapted between different reactions if necessary.

0.3 µl	template
5 µl	dNTPs
10 µl	HF buffer
2 µl	forward primer
2 µl	reverse primer
0.3 µl	Phusion DNA polymerase
30.4 µl	dH_2O

Table (2.13): Standard PCR preparation

Post-PCR DNA purification

PCR products were purified using the NucleoSpin® Extract-II-Kits according to the manufacturer's instructions. During purification the polymerase, oligonucleotides and leftover buffer are removed from the preparation and the DNA concentration in the sample can be adapted according to the amount of elution buffer used.

During purification, DNA binds reversibly to a silica membrane under high salt concentrations. It can then be eluted by TE-buffer and stored ad 4° or -20°C.

Agarose gel electrophoresis (Garoff and Ansorge 1981)

Agarose gel electrophoresis was used to separate DNA fragments of different sizes.

DNAs are negatively charged due to the phosphate groups in their backbone. Thus, DNA fragments migrate to the anode of an electric field. In an agarose gel, the migration rate of linear DNA is inversely proportional to the logarithm of its length (i.e., base count).

For gels used in this study containing 1% agarose (w/v), the optimal range of separation is 0.5 to 7 kilobases. Ethidiumbromide was used to render DNA fragments visible. This substance intercalates itself into DNA and is fluorescent under UV-immision. This method allows detection of DNA quantities lower than 6 ng. A loading dye containing bromphenol blue was used to visualize the samples in the gel.

The 1% agarose gels were prepared by dissolving $2 \times g$ agarose in 200 ml TAE buffer using a microwave. At about 60°C, 5 µl of ethidiumbromide were added and the solution was poured into the casting mold. Pockets for the samples were formed by plastic combs that are removed from the gel after polymerisation. DNA fragments were separated under approximately 10 $\frac{V}{cm}$. To compare sizes, a standard DNA ladder was used.

DNA restriction digest

Type II restriction endonucleases (Pingoud, Wilson, and Wende 2014) are enzymes that are able to hydrolyse phosphodiester bonds at specific sequences in the DNA backbone. These specific restriction sites typically consist of four to eight nucleotides in a palindromic arrangement. Most restriction endonucleases (RE) generate DNA fragments with a free 5'-phosphate- and a 3'-hydroxyl-group after hydrolytic cleavage. Depending on the enzyme, this can result in "blunt ends" (the DNA double strand being cut at the same position) or "sticky ends" (one strand overlapping the other in one or more positions). Restriction enzymes used for this thesis produce fragments with sticky ends and are listed in section 2.1.6.

Preparations of DNA restriction digests (see table 2.14 for an example) were prepared using typically 1 µl enzyme per 1 µg DNA (1 µl enzyme equaling 1 U) and incubated at 37°C for 30-60 minutes. If a plasmid template was used (i.e. methylated DNA), 1 U DpnI was added to the preparation to digest the template (Lacks and Greenberg 1975).

30 µl	purified PCR product
6 µl	10x restriction buffer
1 µl	enzyme 1
1 µl	enzyme 2
22 µl	dH_2O

Table (2.14): Standard DNA restriction digest.

Ligation of DNA fragments

DNA fragments can be inserted into plasmid vectors by enzymes called DNA ligases if both have been digested by REs and present corresponding "sticky ends". A DNA ligase catalyses the formation of phosphodiester bonds between free 5'-phasphate- and 3'-hydroxyl-groups and thus the closing of the DNA backbone.

Vector and insert were purified using the NucleSpin® Extract Kit according to the manufacturer's protocol. For the ligation reaction, a ratio of insert-to-vector of approximately 5:3 was used. A typical preparation of 10 µl would contain 5 µl insert DNA, 3 µl vector DNA, 1 µl T4 DNA ligase and 1 µl T4 ligase buffer containing ATP. The preparations were incubated at room temperature for at least 30 minutes.

Growth and storage of *E.coli*

E.coli were grown either on LB-agar plates or in liquid LB medium containing, if necessary, ampicillin at a concentration of 100 µg/ml. Bacteria were grown for 24 h at 37° C in an incubator and shaken for aeration when liquid LB was used. *E.coli* on Agar plates were stored at 4°C.

For the long-term storage of *E.coli*, glycerol stocks were prepared by centrifuging 0.5 ml of cultured bacteria for 30 s at 1000 g, resuspending the sediment in 0.5 ml of fresh liquid LB and adding 0.5 ml glycerine. The resulting suspension was stored at -80°C.

Transformation of competent *E.coli* (Pope and Kent 1996)

Transformation describes the process of inserting exogenous DNA in prokaryotic cells. To this end, 100 µl chemically competent cells were thawed on ice, mixed with 10 µl of plasmid DNA obtained in a ligation reaction (*vide supra*) and incubated for at least 30 min on ice. To allow efficient DNA uptake, the cells were subsequently heat-shocked at 42°C for 40 s and then plated on LB Agar containing ampicillin using sterile glass beads. After incubating the plated bacteria for 24h at 37°C, a colony screen was performed to identify successfully transformed clones.

E.coli colony screen

Colonies that grew on agar containing ampicillin after transformation were used as template DNA in the PCR preparation. Primers were chosen in a way that PCR could only yield amplified DNA if transformation was succesful. To this end, one used primer would usually bind to a sequence specific to the parental plasmid vector while the second oligonucleotide would be insert-specific.

Screened colonies were re-grown on a master plate to allow further cultivation. Candidate colonies yielding PCR product of the correct length were identified in the screen and single clones were sequenced to further confirm the correct transformation process (sequencing by SeqLab, Göttingen).

Plasmid purification

Plasmid purification in this study was perfomed using commercially available kits. For minipreparations, the NucleoSpin® Plasmid-Kit (Macherey-Nagel) was used. Midiprepa-

rations for larger DNA yields were carried out with the QIAGEN Plasmid Kit Midi 100 (QIAGEN). Both kits were used according to the manufacturer's protocol.

For minipreps (midipreps), 2 (120) ml of overnight *E.coli* culture were centrifuged, re-suspended in buffer containing RNAse-A and lysed in alkaline buffer. The lysed material was subsequently incubated in a neutralization buffer at physiological pH to minimize DNA damages. The preparation was then centrifuged to separate the plasmid DNA from cellular debris.

In minipreps, the plasmid DNA was allowed to bind to a silica membrane, washed multiple times with a buffer containing ethanol and finally eluted with a buffer of 5 mM Tris-HCl at pH 8.5.

In midipreps, the plasmid DNA was allowed to bind in an anion-exchange collumn, washed several times and eluted with a buffer of 1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% propanol [v/v]. The eluted DNA was precipitated with 0.7 volumes of isopropanol, centrifuged, air-dried and redissolved in 200 µl TE-buffer.

Minipreps were used to prepare samples for DNA sequencing while midipreps were used to produce DNA for tranfections of *P. falciparum* cultures.

Measuring sample DNA yield

The DNA content of a sample can me measured by determining the extinction of light at 260 nm wavelength, corresponding to the maximal absorbtion of nucleic bases. An OD_{260} (optical density at 260 nm wavelength) of 1 corresponds to 1 µg/ml of double-strand DNA. In this study, sample DNA yield was determined using a biophotometer after diluting the sample 1:100 with dH_2O against a benchmark of dH_2O.

DNA precipitation

For one transfection of *P. falciparum* culture, about 100 µg of plasmid DNA isolated with a midiprep kit was precipitated by first adding 1/10 vol. of 3 M Sodiumacetate pH 5.2 and subsequently 3 vol. ethanol 100%. The precipitate was then sedimented by centrifugation at $16.000 \times g$ for 5 minutes. After decanting the supernatant the DNA was washed with 70% ethanol, air-dried and redissolved in TE-buffer.

2.2.3 Procedures for *P. falciparum* Cell Culture

Culture of *P. falciparum* blood stages (Trager and Jensen 1976)

To culture *P. falciparum* blood stages, parasites were kept in $14 \times_9 0$ mm Petri dishes at 37°C under low-oxygen conditions (1% O_2, 5% CO_2, 94% N_2). The dishes were kept in separate boxes that were aereated with this custom atmosphere.

Each dish was filled with about 12 ml of RPMI-medium supplemented with AlbuMAX II® to which human erythrocyte concentrate (blood group 0+) was added to a hematocrit of 5% (Cranmer et al. 1997).

To select for transgenic parasites, the antifolate WR99210 (final concentration 10 nM) or the antibiotic blasticidin S (1.5 µg/ml) were used depending on the carrier plasmid.

The replication cycle of the *P. falciparum* blood stage takes approximately 48 h. Thus, in a heterogenous cell culture, parasitemia usually multiplies by factors between 4 and 10 within two days. Parasitemia was estimated every two days by blood smears. Medium was changed in the same interval and the culture was diluted if the estimated parasitemia exceeded 4%. Work with transgenic *P. falciparum* was carried out under biosafety level S3* under the directive of the German Biostoffverordnung.

Preparation of Giemsa-stained blood smears (Giemsa 1904)

Giemsa-stained blood smears were prepared in this study to estimate parasitemia in *P. falciparum* cell culture.

0.5 µl of sedimented erythrocytes were applied to an object slide and smoothened out with a second slide. The erythrocyte now form a thin monolayer on the glass surface. The smear was air-dried and then bathed in methanol for 30s for fixation. Subsequently it was stained in 10% Giemsa solution for at least 15 minutes. After washing with H_2O and air-drying, the smear was analyzed using an optical microscope (Zeiss, standard 20 with 1000-fold magnification). In smears prepared according to this protocol, the erythrocyte cytoplasm appears in a light pink to red. In contrast, the DNA and parasite proteins form complexes with the stain and appear in a strong purple or blue. Parasitemia was estimated by counting 2-3 fields of view and forming the ratio of infected to uninfected red blood cells.

Synchronisation of *P. falciparum* cultures (Lambros & Vanderberg, 1979)

A culture of *P. falciparum* is called sychronous if all parasites are in the same stadium of the intraerythrocytic life cycle. To synchronize a heterogenous cell culture, it was treated with

5% D-Sorbitol. In this concentration, Sorbitol lyses all erythrocytes infected with parasites in trophozoite or schizont stages. Only uninfected red blood cells and those infected with ring stages remain intact.

Synchronisation of heterogenous *P. falciparum* cultures were performed by centrifuging the entire content of one Petri dish at $1.500 \times g$ for 3 minutes, discarding the supernatant, and incubating the cells with 5 vol. of 5% D-sorbitol solution for 5 minutes. Subsequently, the preparation was re-sedimented with the same parameters, washed with fresh RPMI-medium, centrifuged and resuspended in medium. Erythrocyte concentrate and/or selcetion drugs were added if necessary for the continuation of the cell culture.

Transfection of *P. falciparum* cell lines (Wu et al. 1995; Crabb and Cowman 1996; Fidock and Wellems 1997; Crabb et al. 2004b)

The introduction of exogenous DNA into eukaryotic cells is called transfection. A variety of transfection methods including chemical, biological and physical procedures is used for different organisms and cell types. Four membrane bilayers must be crossed in order to transfect *Plasmodium* parasites: the RBC membrane, the PVM, the PPM and the nuclear membrane of the parasite. Until now, only electroporation proved suitable for this task. The short induction of an electric field leads to the temporary perturbance of cellular membranes and allows plasmid DNA to enter into the parasite's nucleus.

Freezing of cultured *P. falciparum*

Freezings of transgenic *P. falciparum* cell lines were prepared for long-term conservation.

A culture containing 5% or more ring stages was sedimented at $1.800 \times g$ for 3 minutes and resuspended in 1 ml "malaria freezing solution" (MFS, see section 2.1.4). These freezings were stored at -80°C.

Thawing of frozen *P. falciparum*

To thaw and re-cultivate stored *P. falciparum* cell lines, freezings were thawed at 37°C in a water bath, centrifuged at $1.800 \times g$ for 3 minutes in a 15 ml falcon tube and resuspended in 1 ml "malaria thawing solution" (MTS, see section 2.1.4) after discarding the supernatant. The suspension was re-sedimented and washed in 1 ml RPMI-medium. After discarding the supernation, the sediment was re-cultured at a hematocrit of 5%. Selection drugs were added after 24 h. In double transfectant cell lines, WR (selecting for the already established

pARL1 plasmid) was added once after 24 h and then omitted until parasites appeared in Giemsa stained blood smears.

Separating different developemental stages of *P. falciparum* via percoll-gradient

For some biochemical assays, it is necessary to purify trophozoite infected erythrocytes in order to minimize background and contamination by hemoglobin and other proteins. To this end, the cellular fraction of a 12 ml *P. falciparum* culture was sedimented at $15.000 \times g$ for 5 minutes in a 2-ml tube on a percoll gradient. The gradient was built up on 550 µl each of 80%, 60% and 40% of percoll solution diluted in RPMI-medium.

Debris and merozoites accumulate at the top and schizonts at the bottom of the 40% fraction, while trophozoites, depending on their age, form accumulations in the 60% fraction. Uninfected RBCs and ring stages sediment with the 80% fraction. Thus, it is possible to discard parts of the cell culture that are not needed for analysis and to obtain highly purified cell populations of one stage.

To perform experiments on puriefied trophozoites, the corresponding fraction of a Percoll gradient was washed three times in 1 ml dPBS and sedimented at 5000 g.

2.2.4 Microscopy

Transmitted-light microscopy

In order to analyse Giemsa-stained blood smears for this study, a transmitted-light microscope was used at 1000-fold magnification with immersion oil.

Live cell imaging by fluorescence microscopy

Live Cell Imaging of transgenic *P. falciparum* cell lines was performed by detecting the luminescence of fluorescence-tagged fusion proteins. Green Fluorescent Protein (GFP, green; Shimonura et al., 1961; Chalfie et al., 1994) and monomeric Cherry (mCherry, red; Shaner et al., 2004) were used as fluorescent protein tags in this study. The luminescence of their fluorophores can be detected without any prestaining making them suitable for studying the localisation of fusion proteins in living cells.

To image transgenic parasite cell lines, 500 µl of *P. falciparum* culture were sedimented at $1800 \times g$ for 3 minutes, the supernatant was discarded and the cellular fraction resuspended in

100 to 200 µl of RPMI-medium or dPBS. If needed, 0.5 µl of 4',6-diamidino-2-phenylindole (DAPI) were added to the initial 500 µl of cell culture and incubated for 5 minutes to stain DNA. Subsequently, 5 µl of the cellular resuspension were prepared for microscopy between an object slide and a cover slip. Using immersion oil, the living cells were analysed by fluorescence microscopy. Images could be taken in four channels (green, red, blue, DIC). Images were edited using CorelDraw 6®.

2.2.5 Biochemical Methods

Preparation of protein extracts from cultured *P. falciparum*

To prepare parasite protein extracts, 5-10ml of parasite cell culture were harvested and trophozoites were purified in a Percoll gradient (see section 2.2.3). The purified cells were pelleted and incubated with 50 to 100 µl freshly prepared saponin 0.015%/1xPBS for 30 minutes on ice and subsequently centrifuged at 16.000 rpm. The supernatant was mixed with 25x protease inhibitor cocktail and 6x SDS buffer. The pellet was resuspended with 2-5 µl of complete protease inhibitor cocktail (Roche) and 50-100 µl of lysis buffer. The extracts were stored at -20°C and analyzed by western blot.

Protein separation by SDS-PAGE (Laemmli 1970)

Proteins were separated using SDS-PAGE (SDS polyacrylamide gel electrophoresis). 4x SDS loading buffer were mixed with the parasite extract and incubated for 5 minutes at 95°C. Subsequently, the mixture was loaded to SDS gels containing 12% or 15% acrylamide. A marker was added to determine the weight of protein bands and the separation was carried out in 200V over 60 minutes in SDS electrophoresis buffer.

Western blot (Towbin, Staehelin, and Gordon 1979)

To detect proteins in polyacrylamide gels, they were transferred onto a nitrocellulose membrane. For this, the membrane and chromatography paper were soaked in transfer buffer. The membrane was placed on top of three layers of paper, the gel was covered on top of the membrane and covered by another three layers of paper. The resulting stack was protected by sponges, secured in the transfection chamber and covered with transfer buffer. The transfer was carried out overnight (ON) at 15 V or at 100 V for 1 h. Subsequently, the membrane

was incubated with blocking solution by rolling for 1h at room temperature (RT). Next, the membrane was incubated either ON at 4°C or for 1 h at RT with the primary antibody diluted in 5 ml of blocking solution. After washing the membrane with about 5 ml PBS for 5 minutes per wash, it was incubated for 1 h at RT with the secondary antibody diluted in blocking solution. Again, the membrane was washed 3-5 times with PBS. Finally, the membrane was incubated with chemiluminescence solution for 2 min and placed between two overhead transparencies inside a developer cassette to be used to expose a film.

Chapter 3

Results

3.1 The export of PEXEL proteins involves a translocation step

It was previously shown that a mDHFR domain fused to the C-terminus of exported proteins can block the export of the resulting construct upon addition of WR. This is based on the fact that WR stabilises the folding of the mDHFR domain, preventing passage of the molecule through translocons requiring unfolding of their substrates (see section 1.3.3). The WR-induced export block was demonstrated using soluble PEXEL proteins (Gehde et al. 2009; Riglar et al. 2013) as well as soluble and integral membrane PNEPs (Heiber et al. 2013; Gruring et al. 2012). Thus, our first aim was to test whether PEXEL proteins with TM domains (TMDs), a class of protein not yet tested in mDHFR assays, also require unfolding in order to be exported.

To this end, we created fusion constructs of the full-length PEXEL proteins PF3D7_-0900900/STEVOR (MC protein with two predicted TMDs, (Przyborski et al. 2005)) and PTP1 (MC protein with one predicted TMD, (Maier et al. 2008)) with C-terminal mDHFR followed by a GFP tag (see Figure 3.1 for schemes of these constructs). For comparison, we created the same constructs with the soluble PEXEL proteins KAHRP (Wickham et al. 2001) and REX3 (Spielmann et al. 2006). All four constructs were expressed in *P. falciparum* blood stages.

The cell line transfected with the STEVOR construct showed strong green fluorescent foci in the cytsosol of infected RBCs, suggesting localisation of the fusion protein to the MCs (Figure 3.1, A) consistent with the known location of STEVOR (Przyborski et al. 2005). Similarly, the PTP1 construct showed a fluorescent pattern suggestive of the protein's previously described localisation at the MCs (Figure 3.1, B) (Maier et al. 2008). KAHRP (knob-

associated histidine-rich protein) has been found to localise to electron-dense foci at the inner surface of the RBC's plasma membrane called knobs (Wickham et al. 2001; Rug et al. 2006). Accordingly, RBCs infected with parasites expressing the KAHRP construct displayed a fluorescence pattern with a multitude of small foci in the RBC periphery (Figure 3.1, C), which is consistent with the construct localising to the knobs (Wickham et al. 2001). Finally, the REX3 construct showed an even distribution of fluorescence in the cytosol of infected RBCs as well as frequent accumulations in the parasite's food vacuole (Figure 3.1, D). This distribution is expected of an exported protein such as REX3 (Spielmann et al. 2006), which is freely soluble in the host cell and thus is re-internalised as the parasite grows and ingests growing portions of the RBC cytosol.

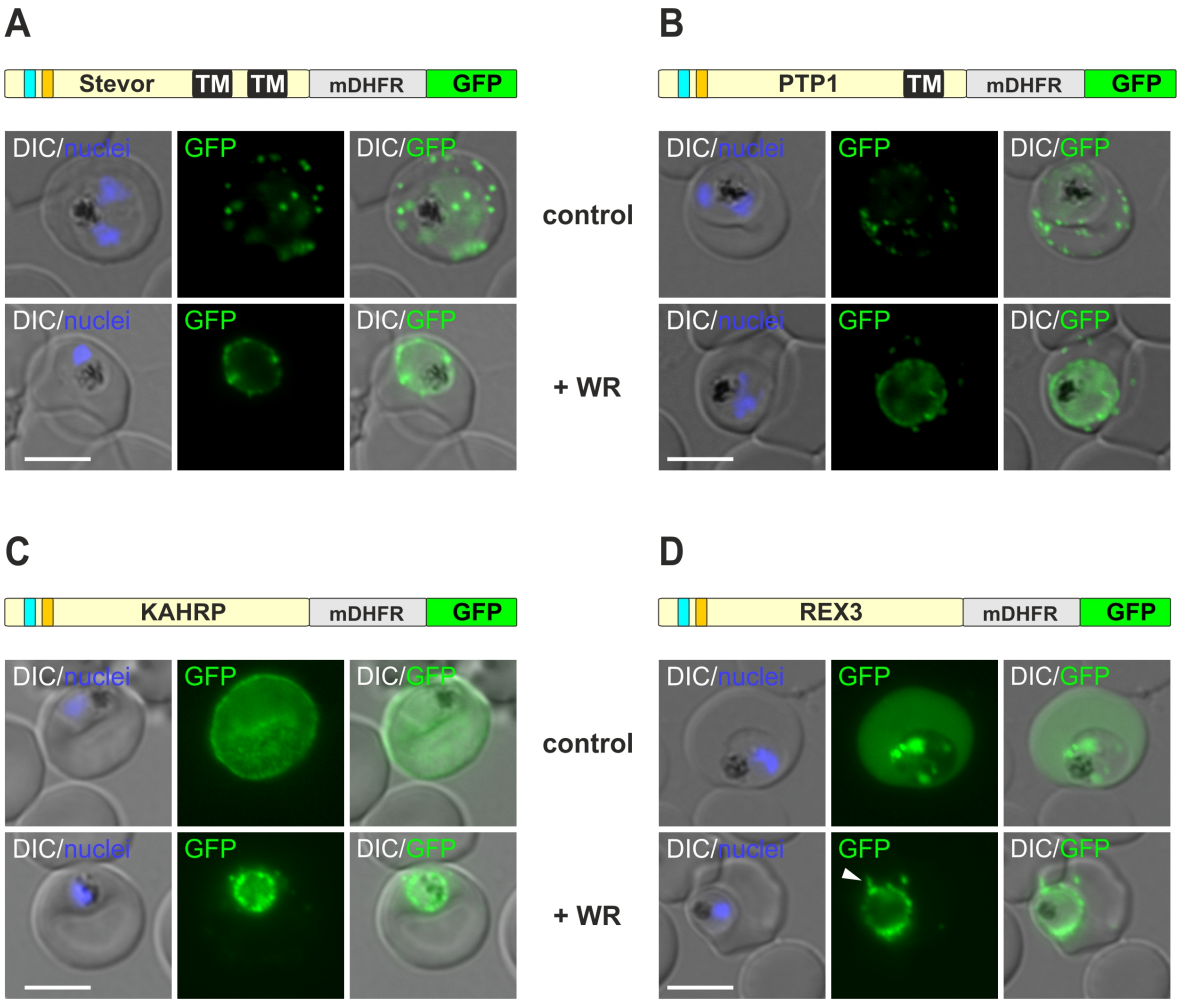

Figure (3.1): Full-length PEXEL proteins with transmembrane domains require unfolding to be exported. (A-D) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel and grown in presence (+WR) or absence of WR (control). White arrowhead: worm-like protrusion; cyan bar: signal peptide; yellow bar: PEXEL-motif; TM: transmembrane domain; size bar: 5 µm.

Upon addition of WR, all four constructs accumulated in the parasite periphery (Figure 3.1, +WR), indicating that the export of those substrates involves a translocation step that is inhibited by the stabilisation of the folded mDHFR domain. In some cells, a varying amount of fluorescence remained visible in the construct's "natural" location even after the addition of WR (for example in Figure 3.1, B). Thus, the export block conferred by WR was not always complete.

It was also noted that the WR-treated cells with REX3-, STEVOR- and KAHRP constructs displayed "rough" fluorescence patterns in the parasite periphery with varying intensity that suggest an uneven distribution of the accumulating arrested export substrates. In some cells, this pattern further included worm-like extensions protruding into the host cell cytosol (Figure 3.1 D, white arrowhead).

In contrast, the peripheral accumulation of fluorescence in WR-treated cells with the PTP1 construct appeared more evenly distributed and showed no protrusions (Figure 3.1), similar to what was previously observed with other mDHFR fusions (Gruring et al. 2012; Heiber et al. 2013). A possible explanation for this behaviour was developed in later experiments (see sections 3.2.5 and 3.3).

3.2 Arrested mDHFR constructs can block the export of co-expressed mCherry-tagged exported constructs

In preliminary experiments the export of PNEPs was studied in a cell line expressing the constructs REX2-GFP-mDHFR together with REX2-mCherry as an internal control (Mesen-Ramirez 2016). REX2 is a short PNEP with one TMD that localises to MCs (Spielmann et al. 2006). Upon incubation with WR, not only the mDHFR but also the mCherry construct appeared to accumulate in the parasite periphery (Figure 3.2 B) (Mesen-Ramirez et al. 2016; Mesen-Ramirez 2016). This was in contrast to a construct where the mDHFR domain and GFP were in a differing order (REX2-mDHFR-GFP, Figure 3.2 A) which did not affect the co-expressed REX2mCherry (Gruring et al. 2012).

We reasoned that this could be evidence that in this configuration (REX2-GFP-mDHFR), export substrates arrested at the host-parasite interface by stabilisation of a mDHFR domain clog the translocation machinery (possibly PTEX), and that this was the cause for the export arrest of the co-expressed reporter without mDHFR (from now on this effect is termed a 'co-block'). If this was the case, it should be possible to test whether different types of exported

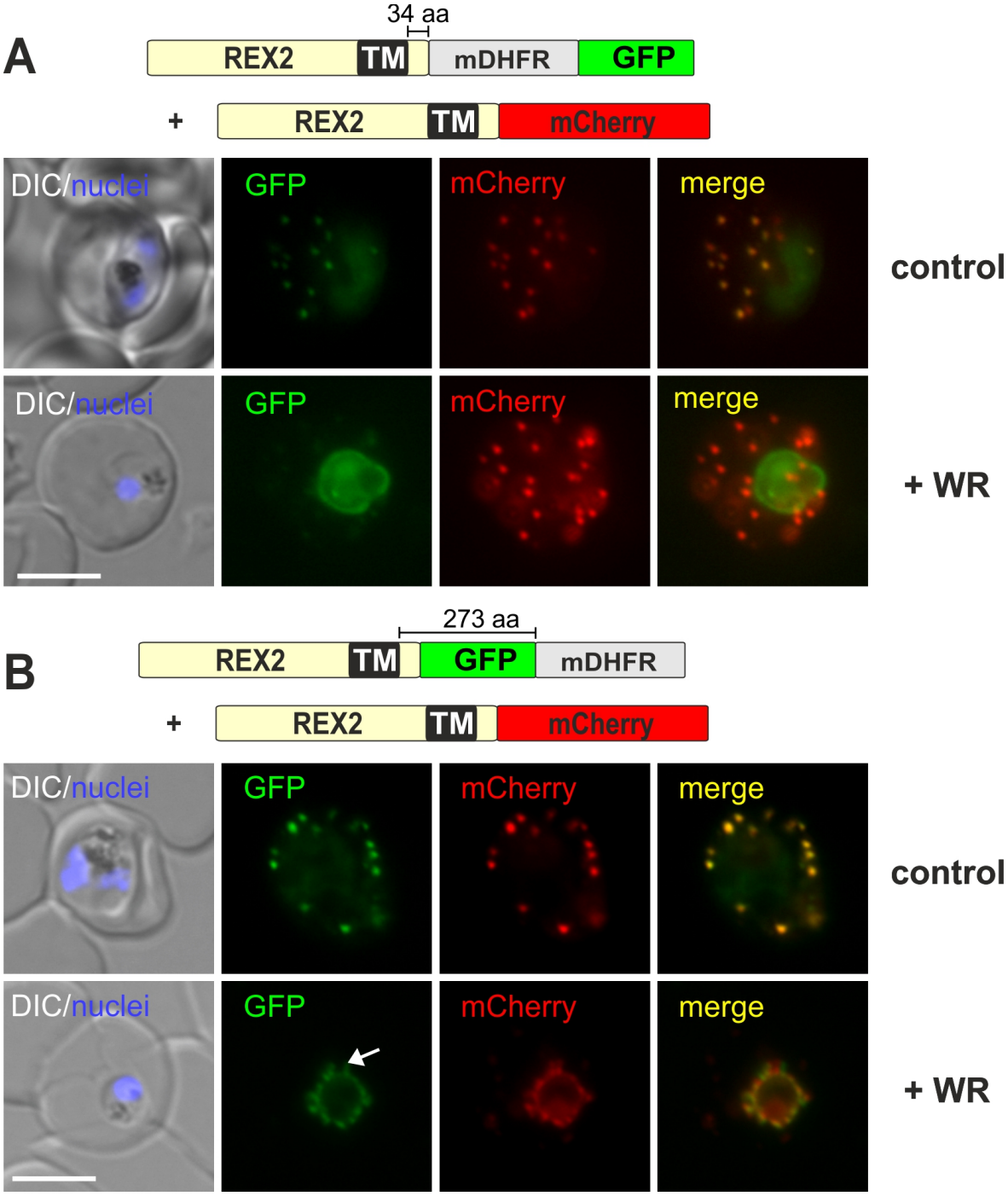

Figure (3.2): Blocking the export of TM-PNEPs fused to mDHFR in a specific configuration can lead to an export block of a co-expressed reporter (co-block). (A, B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above of each panel and grown in presence (+WR) or absence of WR (control). The number of amino acids separating the TMD and the mDHFR moiety is indicated above the mDHFR constructs. Arrow: worm-like protrusion (example); TM: transmembrane domain; size bar: 5 µm Figure modified from (Mesen-Ramirez et al. 2016).

proteins use the same type of translocon by testing which proteins are co-blocked. To this end, we systematically created cell lines expressing mDHFR fusion proteins simultaneously with different exported reporter proteins tagged with mCherry (see section 4.1.2). Our first approach to obtain these cell lines was to conduct two consecutive transfections, one for each construct. This was later switched to an approach that allows the expression of two genes under the control of one promoter on a single plasmid by taking advantage of a "skip peptide", a short viral sequence that leads to a break in the nascent polypeptide chain during translation. The correct expression of two separate constructs through this approach was verified by western blot (data not shown) (Mesen-Ramirez 2016). Furthermore, SBP1-mDHFR-GFP was expressed alongside MSRP6-mCherry in two cell lines, using the skip peptide approach in the first (Figure 3.4 A) and transfection with two plasmids in the second (Mesen-Ramirez 2016). Both cell lines showed similar export phenotypes, confirming the viability of the skip peptide for our experiments.

3.2.1 TM-PNEP constructs with stabilised mDHFR domain can block the export of other TM-PNEPs

To test whether other TM-PNEPs can induce a co-block, two proteins of this class, SBP1 and MAHRP1 (Blisnick et al. 2000; Spycher et al. 2003), were fused to mDHFR and GFP and expressed together with REX2-mCherry. In both resulting cell lines, overlaying green and red fluorescent foci in the RBC cytosol indicated that both constructs were exported and trafficked to the MCs as expected (Figure 3.3, controls). When grown in the presence of WR, the green fluorescent signal of both cell lines accumulated in the parasite periphery (Figure 3.3, +WR), indicating that the export of both SBP1 and MAHRP1 requires an unfolding-dependent translocation step that is inhibited by stabilising the mDHFR moiety. Importantly, when grown in the presence of WR, the red fluorescence signal of the mCherry-reporter in both cell lines was also found to accumulate in the parasite periphery, co-localising with the green signal of the blocked mDHFR construct (Figure 3.3, +WR). This finding suggests that, as outlined in the previous section, some exported mDHFR-fusion constructs can induce a co-block of co-expressed proteins when the unfolding of the mDHFR moiety is prevented by WR. So far, this co-block had been established in cell lines expressing TM-PNEPs fused to mDHFR together with TM-PNEP reporters, although an explanation for the failure of REX2-mDHFR-GFP to induce a co-block (Figure 3.2 A) was only discovered in later experiments (see section 3.3). The observed co-blocks support the notion that TM-PNEPs share the same type of translocon on their route to the host cell.

Often, faint red and green fluorescent foci in the host cell resembling MCs would remain, suggesting a somewhat "leaky" export block (Figure 3.3, white arrowheads). Both the red and green fluorescence surrounding the parasite showed the same "rough" pattern and occasional worm-like protrusions as observed with the PEXEL constructs (see section 3.1).

3.2.2 A TM-PNEP can block the export of a soluble PNEP

In order to test whether a TM-PNEP could induce a co-block of a soluble PNEP, SBP1-mDHFR-GFP was co-expressed with the mCherry-tagged PNEP MSRP6 which localises to MCs but has no transmembrane domain (Heiber et al. 2013). In this cell line, overlaying green and red foci in the RBC cytosol indicated that both constructs were exported and localised to the MCs, as expected. When grown in the presence of WR, both constructs accumulated in the parasite periphery (Figure 3.4 B). Thus, the TM protein SBP1 was able to induce a co-block of the soluble PNEP MSRP6, suggesting that soluble PNEPs are substrates of the same translocation machinery as TM-PNEPs. Furthermore, this suggested that the co-block occurs in the PV or the inner PVM-surface, as soluble proteins are thought to be released into the PV lumen via the default secretory pathway and only then come into contact with the translocon (see section 1.3).

3.2.3 TM-PNEPs can block the export of PEXEL proteins

As our findings so far suggested that the export pathways of PNEPs intersect at a translocation step at the PVM, we next wanted to test whether PEXEL proteins also depend on the same type of translocon to be exported. To this end, SBP1-mDHFR-GFP was co-expressed with mCherry-tagged REX3, KAHRP and STEVOR (see section 3.1) respectively. In all three cell lines, the green fluorescence pattern indicated the expected localisation of the SBP1 constructs to the MCs. Likewise, the PEXEL-reporters showed fluorescence patterns according to their expected localisation (see section 3.1, Figure 3.4 and 3.5). In all cell lines, the reporter was co-blocked upon the addition of WR (Figure 3.4 and 3.5, +WR), indicating that both soluble (REX3, KAHRP) and TM- (STEVOR) PEXEL proteins share one type of translocation machinery with PNEPs.

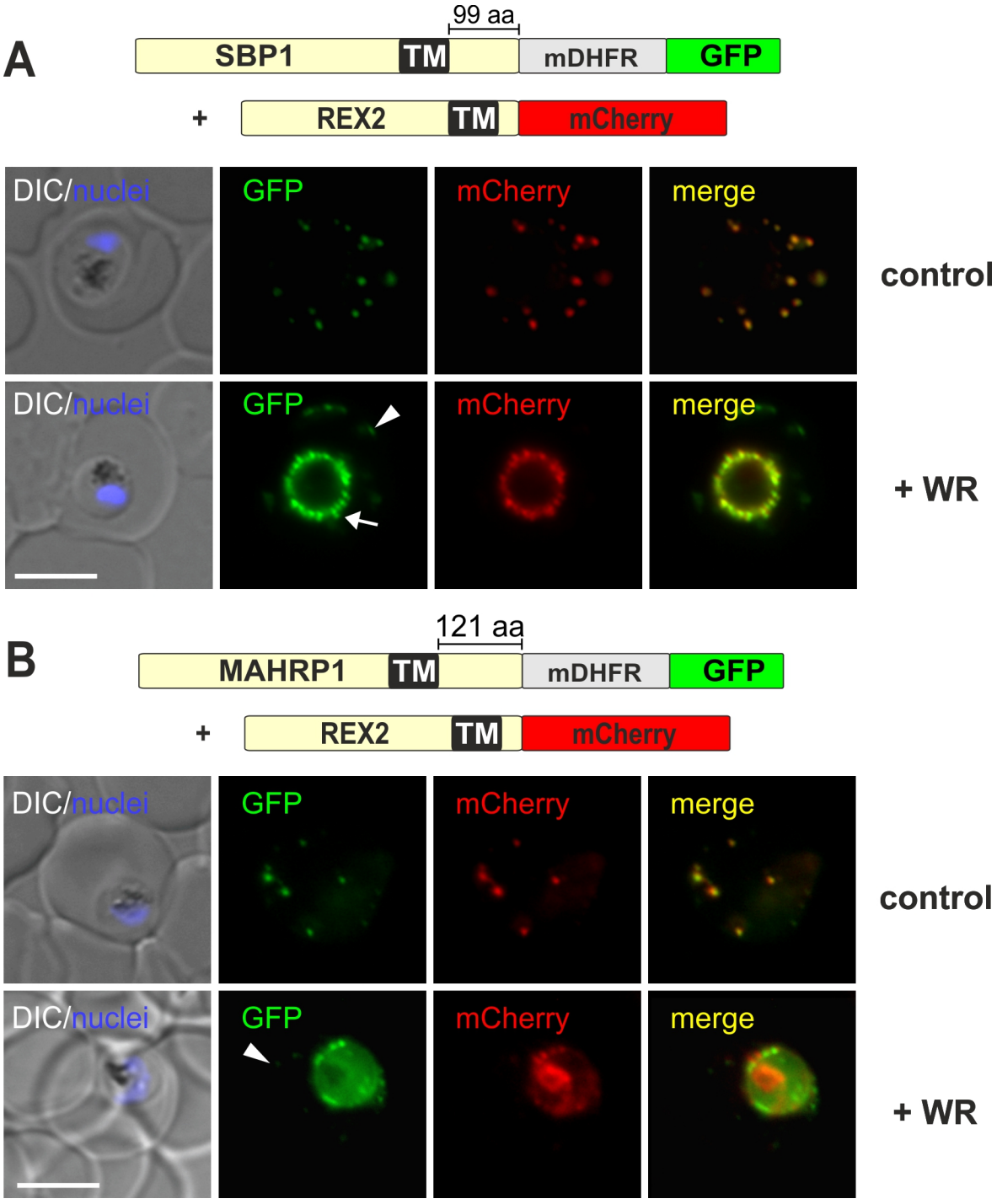

Figure (3.3): Blocking the export of TM PNEPs fused to mDHFR leads to an export block of different co-expressed PNEP reporters (co-block). (A, B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above of each panel and grown in presence (+WR) or absence of WR (control). The number of amino acids separating the TMD and the mDHFR moiety is indicated above the mDHFR constructs. Arrow: worm-like protrusion (example); arrowheads: incomplete block of the mDHFR construct with faint MC-staining (examples); TM: transmembrane domain; size bar: 5 µm Figure modified from (Mesen-Ramirez et al. 2016).

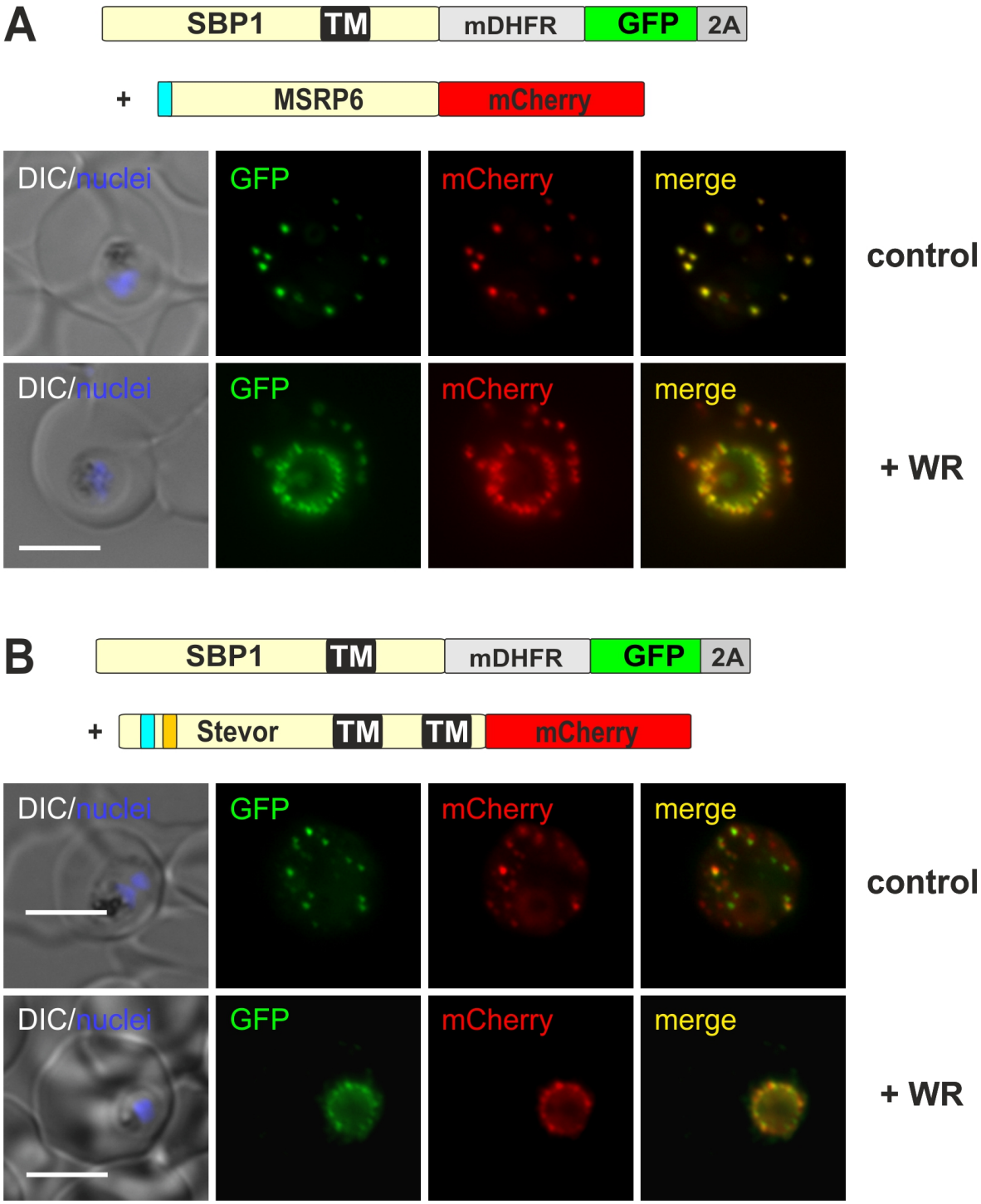

Figure (3.4): A TM PNEP co-blocks a TM PEXEL protein and a soluble PNEP. (A,B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel and grown in presence (+WR) or absence of WR (control). Cyan bar: signal peptide; yellow bar: PEXEL-motif; 2A: skip peptide; TM: transmembrane domain; size bar: 5 µm. Figure modified from (Mesen-Ramirez et al. 2016).

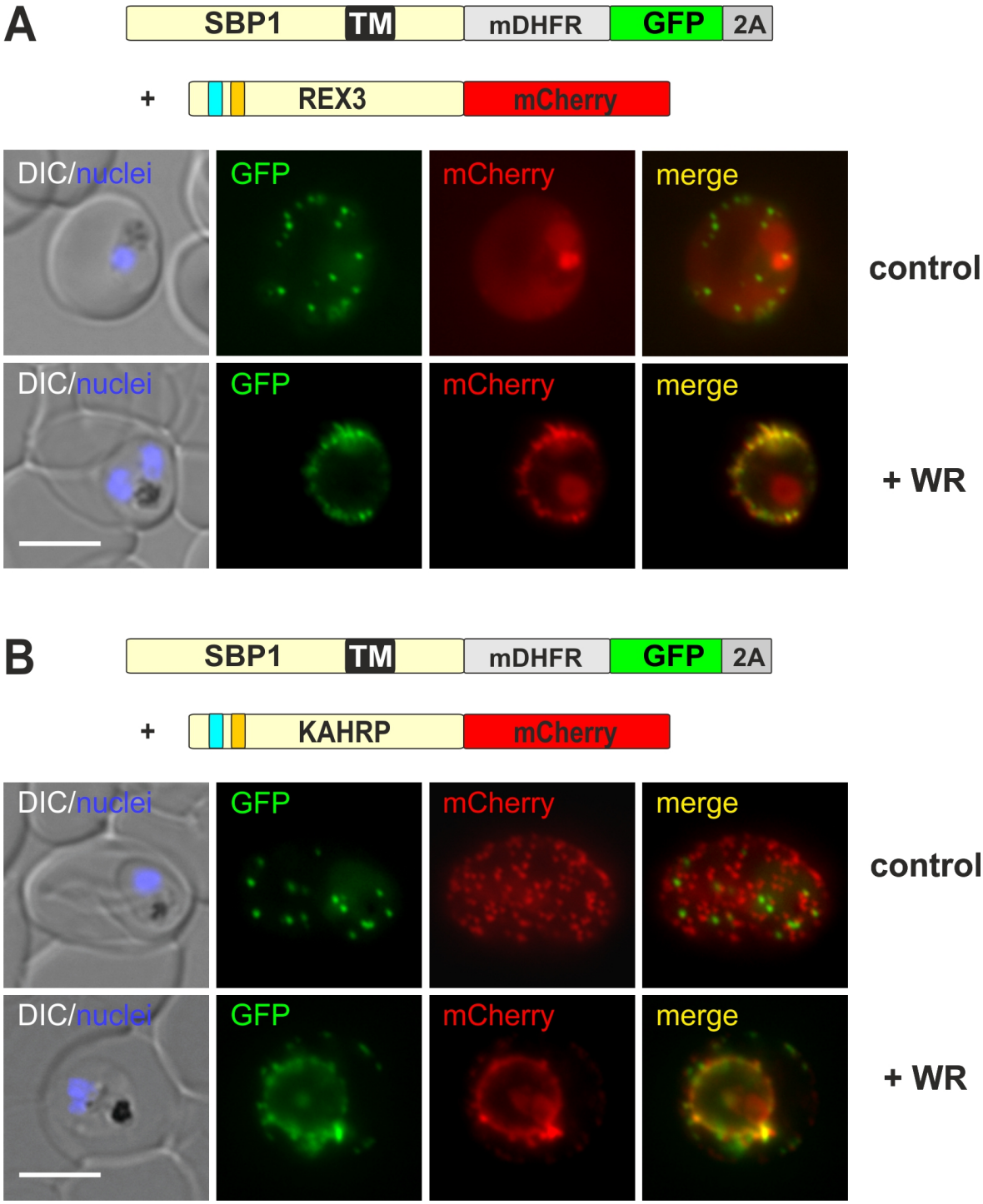

Figure (3.5): A TM PNEP co-blocks soluble PEXEL proteins. (A,B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel and grown in presence (+WR) or absence of WR (control). Cyan bar: signal peptide; yellow bar: PEXEL-motif; 2A: skip peptide; TM: transmembrane domain; size bar: 5 µm. Figure modified from (Mesen-Ramirez et al. 2016).

3.2.4 A soluble PEXEL can block the export of a TM PNEP

So far, mDHFR constructs used to induce co-blocks had been exclusively TM-PNEPs. As those constructs were able to block the export of soluble PEXEL reporters, in the reverse conclusion PEXEL constructs should also be able to induce co-blocks. To verify this hypothesis, a cell line co-expressing the soluble REX3-mDHFR-GFP with REX2-mCherry and a cell line expressing the integral TM PTP1-mDHFR-GFP with REX2-mCherry (see section 3.2.5) were generated.

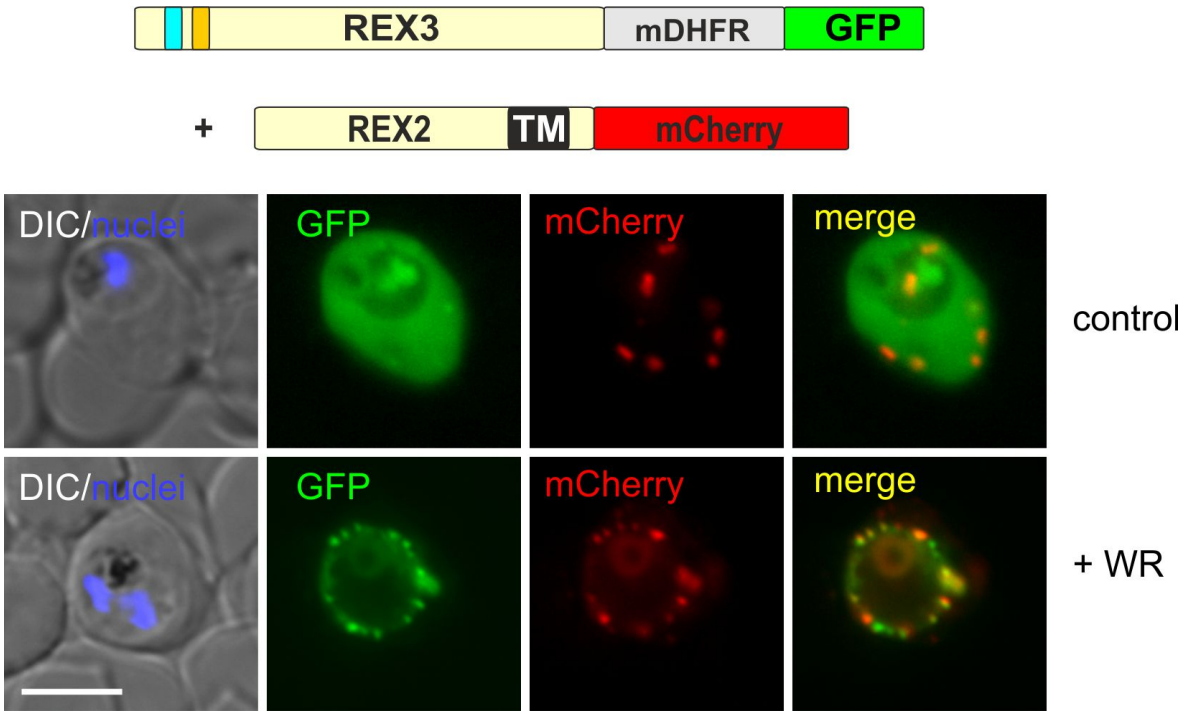

Figure (3.6): A soluble PEXEL construct blocks the export of a TM-PNEP. Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above the panels and grown in the presence (WR+) or absence (control) of WR. Cyan bar: signal peptide; yellow bar: PEXEL-motif; SP: signal peptide; TM: transmembrane domain; size bar: 5 µm.Figure modified from (Mesen-Ramirez et al. 2016).

When grown without WR, both constructs were exported into the host cell as described in the previous sections (Figure 3.6, control). When grown in the presence of WR, the REX3 construct induced a co-block of the REX2-mCherry reporter, with the two fluorescence signals accumulating in an overlaying pattern in the parasite periphery (Figure 3.6, +WR). The co-block was "leaky" with some of the mCherry-signal reaching the host cell. Mobile "worm-like" protrusions as described in section 3.1 were frequently observed.

These findings strengthened our notion that soluble and TM proteins with and without PEXEL all use the same translocon on their way to the host cell and that this translocon facilitates the transport of unfolded substrates across the PVM.

3.2.5 PTP1-mDHFR-GFP fails to induce a co-block

The cell line co-expressing PTP1-mDHFR-GFP and REX2-mCherry showed the expected red-and-green staining of MCs in the host cell.

When the cells were grown in the presence of WR, the arrest of the green fluorescing construct was observed as a smooth circumference surrounding the parasite as described in section 3.1. In contrast, REX2-mCherry, which lacked the mDHFR moiety, continued to be exported to the MCs (Figure 3.7 A). This indicated similar properties of PTP1-mDHFR-GFP to REX2-mDHFR-GFP, which also did not induce a co-block (Figure 3.2) (Gruring et al. 2012).

3.3 Co-blocking ability of TM proteins depends on the distance between TM- and mDHFR domain

Both PTP1 and REX2 have a single TMD followed by a short C-terminus (27 amino acids (AA) in PTP1, 34 AA in REX2). A co-block was first observed induced by REX2-GFP-mDHFR, in which GFP acts as a "spacer" between the TMD of REX2 and the mDHFR moiety, while no co-block was observed in the configuration REX2-mDHFR-GFP (see section 3.2). In REX2-GFP-mDHFR, the distance between TMD and mDHFR moiety (from now on termed "spacer") is 273 amino acids. The shortest spacer in a TM construct tested so far which was able to induce a co-block was in SBP1-mDHFR-GFP with 99 amino acids (see section 3.2, Figure 3.3 A). We hypothesised that the length of the spacer alone determines whether a construct can induce a co-block and aimed to verify this hypothesis by elongating the spacers of originally non co-blocking constructs.

A cell line expressing REX2 fused to three additional REX2-C-termini followed by mDHFR-GFP (REX2-3C-mDHFR-GFP, the abbreviation "3C" will from now on refer to three repeated REX2-C-termini) along with a REX2-mCherry reporter was generated, as well as

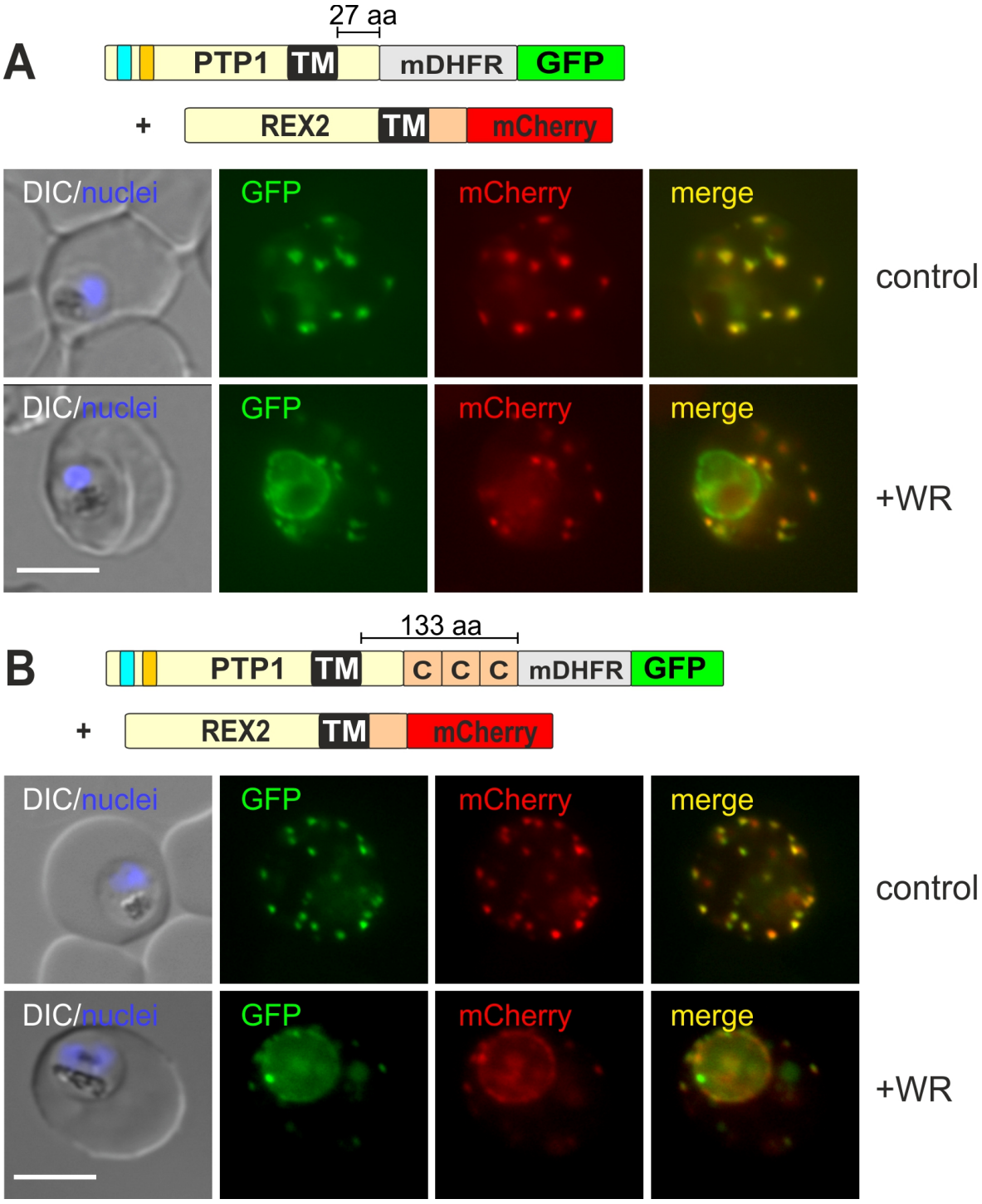

Figure (3.7): Only constructs with a long distance between TMD and mDHFR moiety induce a co-block when grown in the presence of WR. (A,B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel and grown in presence (+WR) or absence of WR (control). The number of amino acids separating the TMD and the mDHFR moiety is indicated above the construct. Cyan bar: signal peptide; yellow bar: PEXEL-motif; TM: transmembrane domain; C: REX2-C-terminus (3×C ≙ 106 amino acids); size bar: 5 µm. Figure modified from (Mesen-Ramirez et al. 2016).

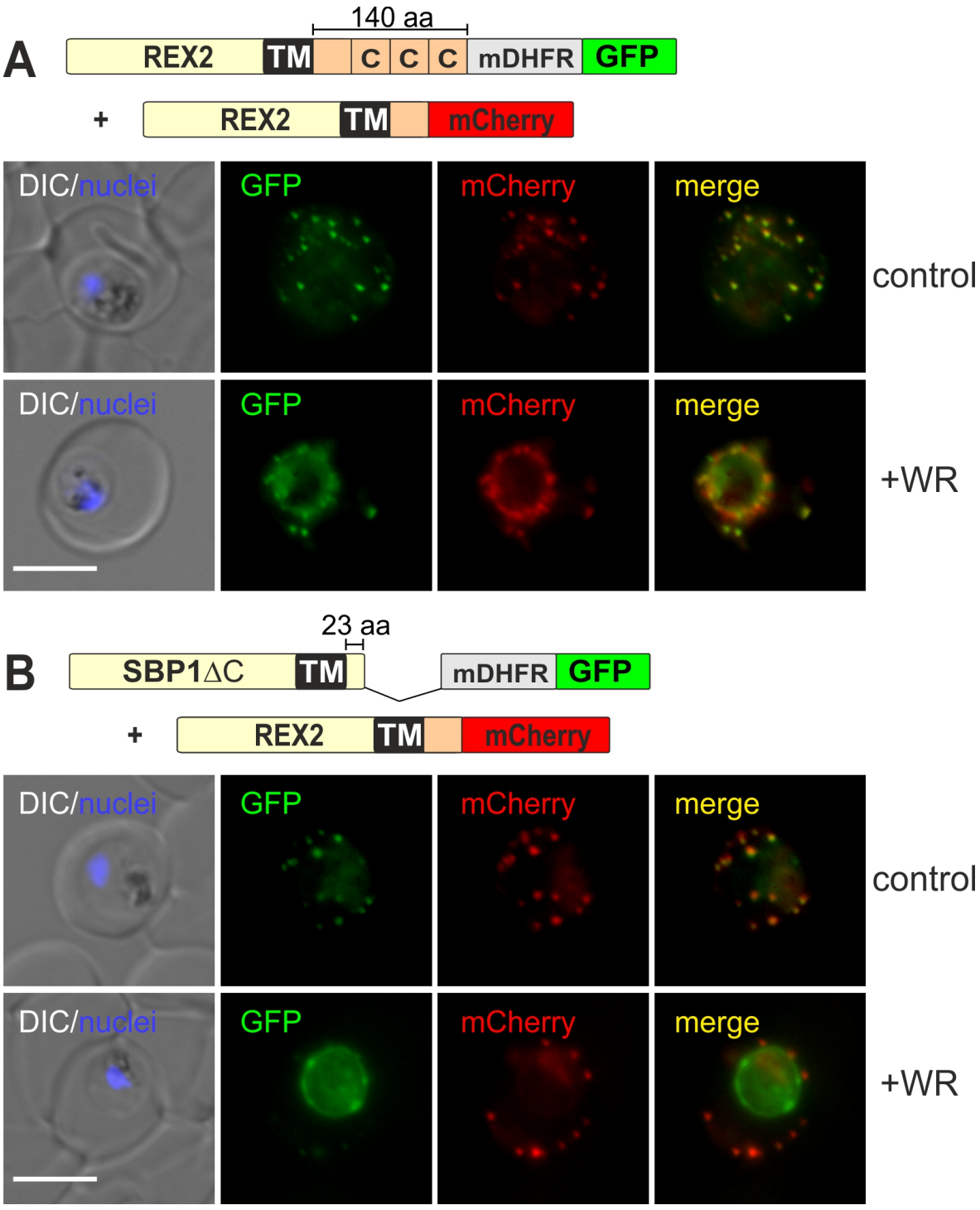

Figure (3.8): Only constructs with a long distance between TMD and mDHFR moiety induce a co-block when grown in the presence of WR. (A,B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel and grown in presence (+WR) or absence of WR (control). The number of amino acids separating the TMD and the mDHFR moiety is indicated above the construct. C: REX2-C-terminus (3×C ≙ 106 amino acids); size bar: 5 µm. Figure modified from (Mesen-Ramirez et al. 2016).

a second cell line expressing PTP1-3C-mDHFR-GFP and REX2-mCherry. This extends the distance between TM- and mDHFR domain from 34 to 140 amino acids in the REX2 construct and from 27 to 133 amino acids following the PTP1 TMD. In both cell lines, the elongation of the apcer in the mDHFR constructs did not interfere with their export or trafficking to the MCs, as the green fluorescence signal co-localised with the foci of the mCherry-reporter in the RBC cytosol (Figure 3.7 B, Figure 3.8 A). When grown in the presence of WR, the red fluorescence signal accumulated together with the green signal in the parasite periphery in both cell lines (Figure 3.7 B, Figure 3.8 A). This demonstrates that the elongation of the spacer between the TMD and the mDHFR moiety by 106 amino acids was sufficient to render REX2 and PTP1 able to induce a co-block.

With these findings supporting our theory that the distance between TM- and mDHFR domain is important for the co-blocking ability of a construct, we wanted to test whether shortening the C-terminus of a co-blocking TM protein would abrogate its co-blocking ability. To this end, a construct of a C-terminally truncated SBP1 fused to mDHFR-GFP (SBP1ΔC-mDHFR-GFP) was expressed alongside REX2-mCherry. The truncation reduced the length of the spacer to 23 amino acids. This construct was exported to the MCs as expected and arrested in the parasite periphery when cells were grown in the presence of WR. In agreement with our hypothesis, no co-block was observed as the mCherry-reporter lacking a mDHFR moiety continued to be exported to the Maurer's Clefts when this cell line was grown in the presence of WR (Figure 3.8 B).

3.4 Double transfectants with soluble proteins

From our previous results, we expected that a soluble PEXEL-mDHFR construct would also block a soluble PEXEL-reporter. To test this, a cell lines co-expressing KAHRP-mDHFR-GFP with KAHRP-mCherry and one co-expressing KAHRP-mDHFR-GFP REX3-mCherry were generated. The KAHRP constructs showed a fluorescence distribution typical for knobs, while the REX3 construct showed the even distribution in the RBC cytosol with an accumulation in the food vacuole that is expected of a soluble exported protein (Figure 3.9, controls). However, while in both cell lines the mDHFR construct was readily arrested in the parasite periphery when the cells were grown in the presence of WR, the behaviour of the mCherry reporters showed a surprising variability in their phenotype.

In the cell line expressing the KAHRP-mCherry reporter, co-blocks were observed frequently (see for example Figure 3.9 A, +WR, first panel), but where often very "leaky", with

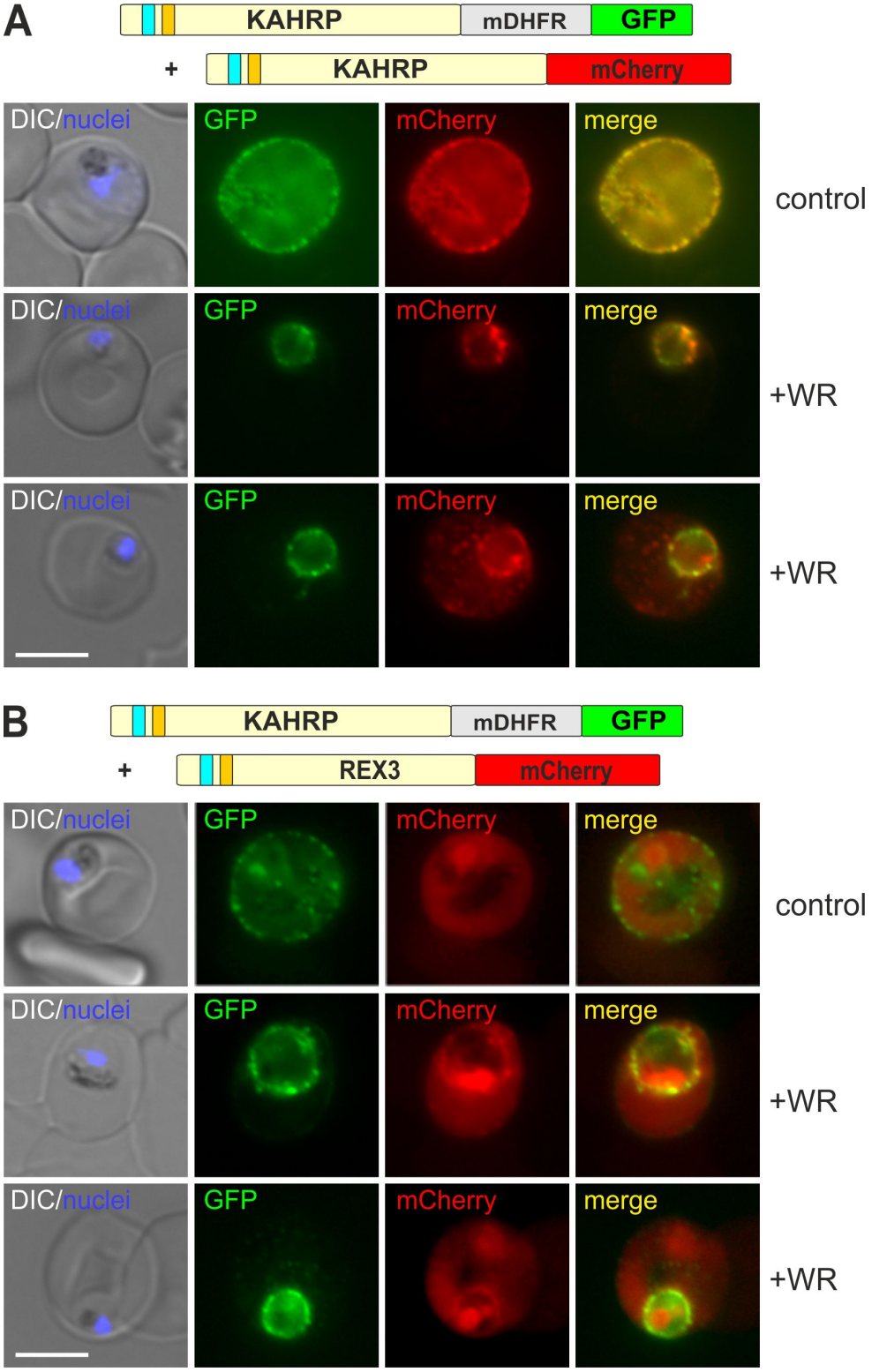

Figure (3.9): Co-blocks between soluble proteins appear to be less effective than when a TM protein is involved. (A,B) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel and grown in presence (+WR) or absence of WR (control). Examples of varying +WR-phenotypes are depicted in two panels, respectively. Cyan bar: signal peptide; yellow bar: PEXEL-motif; size bar: 5 µm.

a substantial amount of red fluorescence signal displaying the typical knob-pattern (see for example Figure 3.9 B, +WR, second panel). In some cells, an accumulation of the mCherry reporter in the parasite periphery was barely visible, despite a clear block of the mDHFR construct.

In the cell line expressing the REX3-mCherry reporter, observable co-blocks were rare and usually very "leaky" (Figure 3.9 B, WR+). The overall red fluorescence intensity appeared to be extraordinarily low (see for example Figure 3.9 B, +WR, second panel; note the heavily boosted red signal), making the evaluation of this cell line difficult.

Taken together, these findings suggest that the co-block induced by a soluble mDHFR construct on another soluble reporter appears to be less effective than when at least one TM protein is involved (see section 4.1.4.

3.5 Bovine pancreatic trypsin inhibitor (BPTI) as a tool to narrow down the model of protein translocation

A different approach to study translocation processes recently adapted for *P. falciparum* in our lab is the utilisation of BPTI as a foldable domain (Mesen-Ramirez et al. 2016; Mesen-Ramirez 2016). BPTI is irreversibly stabilised in a folded state by three intramolecular disulfide-bridges that form under oxidising conditions (Vestweber and Schatz 1988). While the parasite cytosol is considered to be a reducing environment, the PV is thought to be oxidising (Kehr et al. 2010; Kasozi et al. 2013). It was demonstrated that the export of BPTI constructs is unimpeded if the cysteine residues necessary for disulfide bridge formation are mutated (Mesen-Ramirez et al. 2016; Mesen-Ramirez 2016). Any observed export phenotype of BPTI constructs is thus solely a result of the folding of the moiety.

To test this approach, three cell lines were generated, each expressing one of the MC-resident TM proteins REX2, PTP1, or SBP1, fused to a BPTI moiety followed by a GFP-tag. In the cell lines expressing REX2-BPTI-GFP and PTP1-BPTI-GFP, an accumulation of green fluorescence in the parasite periphery was visible, while a faint staining of MCs was also visible (Figure 3.10 A and C). This behaviour is compatible with the BPTI moiety forming disulfide-bridges in the PV, blocking the translocation of the construct across the PVM.

Strikingly, the cell line expressing SBP1-BPTI-GFP displayed a fluorescence pattern with typical MC-foci in the RBC cytosol and no peripheral accumulation, indicating that the BPTI

moiety did not interfere with the export of this construct (Figure 3.10 E). This was surprising, as the export of SBP1 had already been shown to be unfolding-dependent (see section 3.2).

3.5.1 BPTI only interferes with the export of TM proteins when close to the TMD

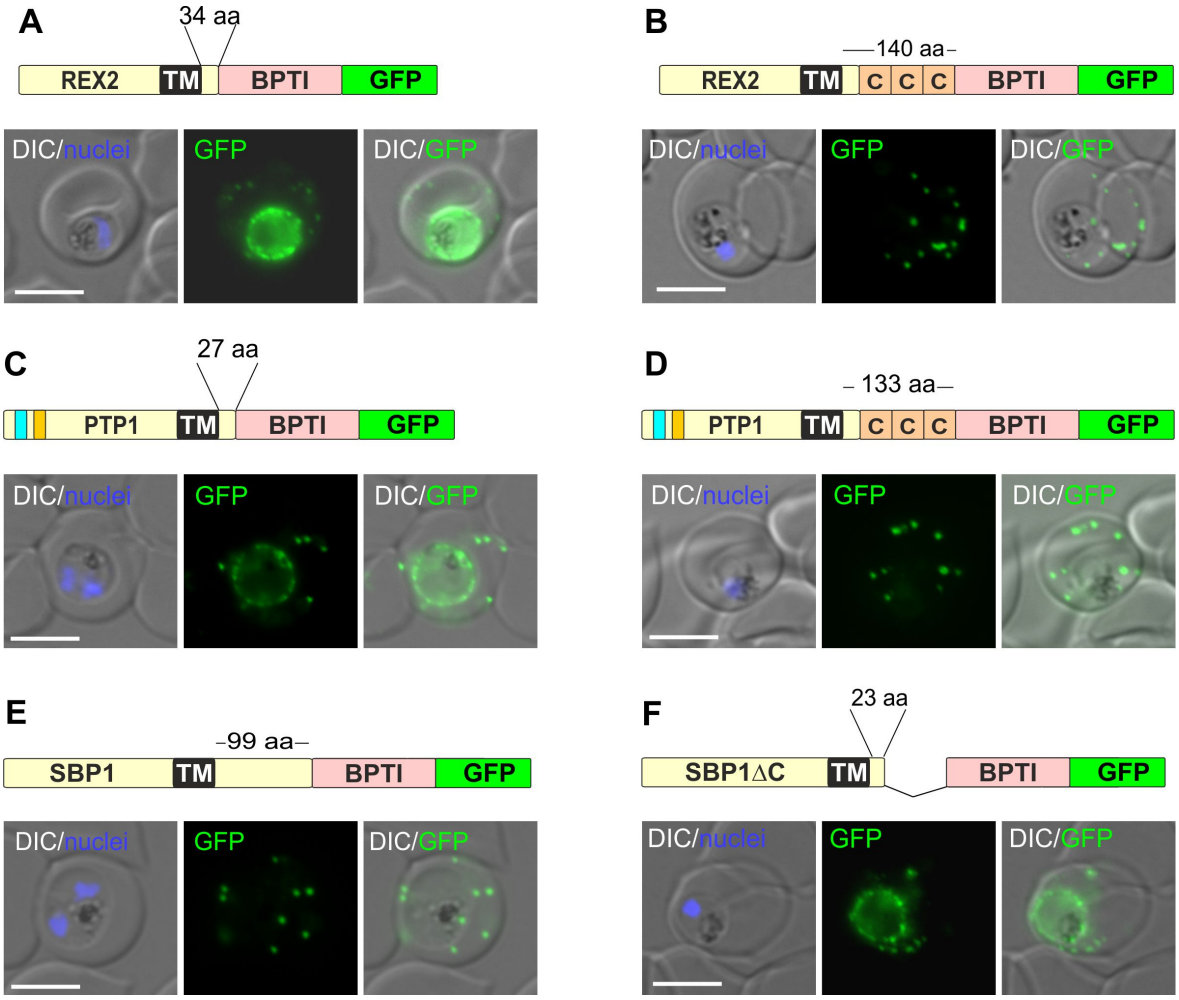

Figure (3.10): A BPTI moiety fused to exported TM proteins only affects export if it is close to the TMD. (A-F) Representative images of live *P. falciparum* parasites expressing the constructs shown schematically above each panel. The spacer lengths are indicated in aa (amino acids). Cyan bar: signal peptide; yellow bar: PEXEL-motif; C: REX2-C-terminus (3×C ≙ 106 amino acids); size bar: 5 µm. Figure modified from (Mesen-Ramirez et al. 2016).

Bearing in mind that in mDHFR constructs the distance between their TMD and the mD-

HFR moiety played a role in their co-blocking ability, we reasoned that likewise the length of the spacer between TMD and BPTI moiety could determine whether BPTI interfered with a contruct's export or not. To test this hypothesis, we generated cell lines expressing constructs in which we extended the spacer of REX2 and PTP1 by 106 amino-acids as described in section 3.3, resulting in the constructs REX2-3C-BPTI-GFP and PTP1-3C-BPTI-GFP. Indeed, both constructs with elongated spacers were exported to the MCs without any peripheral accumulation (Figure 3.10, B and D). This confirmed the notion that BPTI only interferes with export when the distance to the TMD is short.

In order to further substantiate these findings, a cell line expressing a C-terminally truncated version of SBP1 followed by BPTI-GFP was generated. In agreement with our previous observations, this construct showed the typical pattern of peripheral accumulation with a faint staining of MCs. This indicates that the shortening of the spacer from 99 to 23 amino acids leads to the exposure of the BPTI moiety to the PV lumen, whereupon it folds and subsequently interferes with the construct's export (Figure 3.10, F).

3.5.2 Export of a soluble construct is inhibited by BPTI

According to the common model of protein export, a soluble exported protein fused to BPTI should be arrested in the PV, as it would be completely exposed to the PV environment as soon as a secretory vesicle fuses with the PPM to unload its cargo.

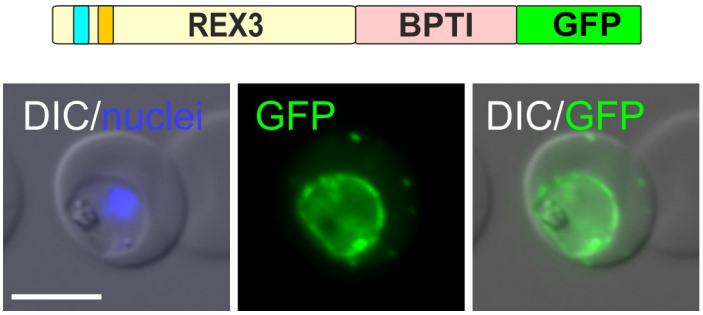

Figure (3.11): A BPTI moiety interferes with the export of a soluble construct. Representative images of a live *P. falciparum* parasite expressing the construct shown schematically above the panel. Cyan bar: signal peptide; yellow bar: PEXEL-motif; size bar: 5 µm. Figure modified from (Mesen-Ramirez et al. 2016).

To verify this, we created a cell line expressing a construct of the soluble PEXEL protein REX3 fused to BPTI-GFP. As expected, the construct accumulated in the parasite periphery (Figure 3.11). In addition faint fluorescence was also observed in the host cell, suggesting some leakiness of the phentoype.

Chapter 4

Discussion

4.1 Major findings

4.1.1 A translocation step is a unifying feature of protein export in *P. falciparum*

Previous studies demonstrated that soluble PEXEL proteins (Gehde et al. 2009; Riglar et al. 2013) as well as soluble (Heiber et al. 2013) and TM PNEPs (Gruring et al. 2012) require an unfolding-dependent translocation step to reach the host cell. The export of constructs consisting of these types of proteins fused to a mDHFR domain could be blocked by the addition of WR, which prevents the unfolding of the mDHFR moiety. In this study, we expressed full-length PEXEL proteins fused to mDHFR and GFP in *P. falciparum* blood stages, covering soluble proteins (REX3, KAHRP), as well as PEXEL proteins with a single predicted TMD (PTP1) or two predicted TMDs (STEVOR). All of these constructs were expressed and trafficked to their previously characterised subcellular localisations (Wickham et al. 2001; Spielmann et al. 2006; Przyborski et al. 2005; Maier et al. 2008). Importantly, all of these constructs were arrested in the parasite periphery when cells were grown in the presence of WR, confirming that TM PEXEL proteins also require an unfolding-dependent translocation step in order to reach the host cell (see section 3.1). This requirement is thus a unifying feature of all types of proteins investigated so far, including soluble as well as integral TM PEXEL proteins and PNEPs.

4.1.2 Simultaneous expression of two exported constructs

In this thesis, several experiments required the simultaneous expression of two exported constructs in one cell line. Our initial approach to obtain these cell lines was to conduct two successive transfections (each with a different plasmid) that eventually yield parasites carrying a pARL1-plasmid with the mCherry-tagged reporter and a pARL2-plasmid with the mDHFR-GFP construct (see section 2.1). This approach is very time consuming for the following reasons: First, a cell line carrying the first transfected plasmid needs to be established before the second transfection can be performed. Due to the long time required to establish a transfected parasite line, the second transfection adds several weeks before the cell line can be analysed. Second, WR was used as a selection drug for the pARL1-plasmid. Because WR is also the ligand which leads to mDHFR stabilisation, protein export of the mDHFR construct was inhibited after the second transfection step. In the course of this study, it became apparent that the arrest of mDHFR constructs interferes with the process of protein export as a whole which significantly impairs parasite growth (Mesen-Ramirez et al. 2016). This effect could only partially be ameliorated by intermittently removing WR from the cell culture during the selection process. Lastly, plasmid segregation during the parasite's blood stage replication is inefficient, i.e. only a fraction of new parasites inherit a plasmid from their "parent" cell (O'Donnell et al. 2002). As a theoretical consideration, selecting for a second plasmid could further slow the growth of the culture, because in every generation all parasites that have lost either of the two plasmids are eliminated. However, we did not verify this consideration experimentally.

To avoid these problems, our approach was switched to the expression of two individual protein constructs from a single expression cassette on a pARL2-plasmid by separating them using a viral 2A-type "skip peptide" (Donnelly et al. 2001; Szymczak et al. 2004; Straimer et al. 2012). After the feasibility of this approach was established (see section 3.2), it became the method of choice to co-express two proteins at the same time (Mesen-Ramirez et al. 2016), circumventing the requirement to conduct two consecutive transfections and the problem of the detrimental effect of WR on parasite growth, as pARL2 is selected for by BSD.

4.1.3 Export pathways converge at the translocation step across the PVM

Although *P. falciparum* cell lines expressing exported mDHFR constructs simultaneously with a second fluorescence-tagged exported protein were analysed in previous studies, the phenomenon of co-blocking has not been described before. This was due to the fact that in those studies, only such mDHFR constructs that we now found to be non-co-blocking (with a short spacer) or weakly co-blocking (double transfectants with two soluble constructs) were used (Gruring et al. 2012; Heiber et al. 2013). We exploited the previously unknown ability of exported constructs arrested in the parasite periphery by mDHFR stabilisation to block the export of other proteins to demonstrate that the translocation step across the PVM is facilitated by the same mechanism for all types of exported proteins (Mesen-Ramirez et al. 2016).

To this end, we created a series of cell lines expressing exported mDHFR-GFP constructs alongside an exported mCherry-tagged reporter. This way, we showed that TM-PNEPs could block the export of other TM-PNEPs as well as soluble and TM-PEXEL proteins, that TM-PEXEL proteins could block the export of TM-PNEPs and, importantly, that also a soluble PEXEL protein could block the export of a TM-PNEP (see section 3.2.4).

The observed co-block between different kinds of proteins strongly suggests that the arrested mDHFR constructs jam the molecular translocation machinery, preventing other proteins from being exported. A previous study suggested that TM-PNEPs undergo two translocation steps, one at the PPM followed by a second one at the PVM, in order to reach the host cell (Gruring et al. 2012). Soluble exported proteins are thought to be released into the PV-lumen via the default secretory pathway. Thus, the fact that a soluble mDHFR construct could induce a co-block supports the notion that the the converging point of export pathways is equivalent to the second translocation step of TM proteins. This is consistent with PTEX on the luminal side on the PVM being the location where the co-block occurs.

Indeed, colleagues in our lab were able to show that arrested co-blocking substrates interact with the PTEX-component EXP2 (Mesen-Ramirez et al. 2016). It was also demonstrated that in cells with a co-block that was induced by an early expressed construct, not only the export of co-expressed constructs but also that of endogenous proteins is blocked, and that this global block of protein export leads to an arrest of parasite development (Mesen-Ramirez et al. 2016). Similarly, two other studies demonstrated that parasites in which protein export is abrogated by knock-down of PTEX components become arrested in early trophozoite stages (Elsworth et al. 2014; Beck et al. 2014).

Taken together, these data indicate that co-blocking constructs with a stabilised mDHFR

domain are able to jam parts of the PTEX complex in a way that globally impedes its translocation ability.

4.1.4 The co-block between two soluble PEXEL proteins is inefficient

For this thesis, two double transfectant cell lines expressing soluble PEXEL-mDHFR constructs alongside soluble mCherry-tagged reporters were generated (section 3.4). While co-blocks induced by the mDHFR constructs in cells grown in the presence of WR were observed in both cell lines, the oberved phenotype appeared to be more leaky than in experiments where at least one of the co-expressed constructs was an integral membrane protein. The co-block induced by KAHRP-mDHFR-GFP on REX3-mCherry appeared to be particularly weak.

It is possible that soluble co-blocking proteins jam PTEX in a way that inhibits the translocation of TM proteins more efficiently than that of soluble substrates. Accordingly, a previous study with double-transfectants expressing soluble PNEP-mDHFR constructs did not report a co-blocking phenotype (Heiber et al. 2013).

We observed that the cell line expressing REX3-mCherry as a reporter retained the red fluorescent construct after a prolonged period without drug selection (> 4 weeks, data not shown). This gave rise to the consideration that our observations could be the result of an unintended recombination event of the pARL1 construct whereby the mCherry-tagged gene integrated into the genomic locus. This could have led to the mCherry construct being expressed and exported before PTEX was saturated with arrested mDHFR constructs. These latter constructs were expressed episomally on the pARL2-plasmid under the control of the *crt*-promoter, which leads to transcription starting in early trophozoite stages. Co-blocks were observed more often in older parasite stages (see for example Figure 3.9 B, +WR, first panel), which could have been the result of the continuous accumulation of mCherry-reporter after the onset of the mDHFR construct's transcription. However, we did not test these cell lines for genomic integration events, and the prolonged retention of a plasmid without drug selection by transfected parasites has previously been described in cell lines that have been exposed to continuous drug cycling (O'Donnell et al. 2001). It also can not be excluded that the weak co-blocking ability might be due to some inherent property of KAHRP.

While the cause for the leakiness of the observed phenotypes remains unclear, the fact that weak co-blocks were frequently observed in these two cell lines suggests that our notion of PTEX being the site where the co-block is induced also suits these types of substrates.

4.1.5 Refined models of protein translocation based on different observed export phenotypes

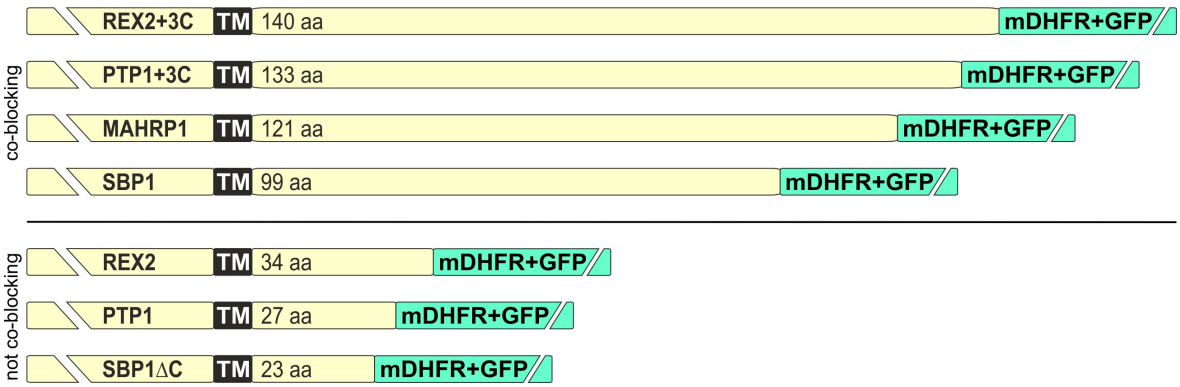

Figure (4.1): Comparing the distance between TM domain and mDHFR moiety of different constructs. The spacers of different tested constructs are depicted proportionally to their length indicated in number of amino acids. The remaining protein features including the TM domain (TM) are depicted schematically. The two observed co-blocking phenotypes are dependent on the spacer length, with the critical length separating the two phenotypes lying between 34 and 99 residues (indicated by the black line). Note that, when mDHFR is replaced by BPTI, the two observed export phenotypes (long spacer: normal export; short spacer: partially blocked export) are likewise separated by a critical spacer length between these two tested lengths.

Remarkably, not all mDHFR constructs were able to induce a co-block. We observed that in cells expressing constructs with a short distance between the TMD and the mDHFR moiety (in this thesis referred to as "spacer"), co-expressed fluorescent reporter constructs were exported normally in the presence of WR (section 3.3). We verified that this failure to induce a co-block was due to the short spacer by creating cell lines with artificially elongated and shortened spacers (section 3.3). An overview of the different spacer lengths tested is given in Figure 4.1. Although we did not systematically search for a minimum spacer length for co-blocking constructs, according to our findings this critical length lies between 34 and 99 amino acids.

Previous studies have demonstrated that the TMD of exported proteins contains information which is necessary to target them to the host cell (Saridaki et al. 2009; Haase et al. 2009; Gruring et al. 2012). Thus it is possible that some kind of receptor inside the PV needs to make contact with the TMD to route a protein through PTEX. Such a receptor could be part of PTEX itself or some not yet identified accessory factor. If thus the TMD serves as a kind of export signal, a model for our two observed phenotypes in mDHFR constructs could resemble the import mechanism of mitochondria.

Mitochondria are surrounded by two membranes, each of which holds a protein translocation complex: the TOM (translocon of the outer membrane) and TIM (translocon of the inner membrane) complexes. Mitochondrial matrix proteins are synthesized in the cytosol and targeted to the mitochondrium by an N-terminal presequence which is cleaved once the unprocessed protein (hereafter called preprotein) has reached the matrix (Matouschek, Pfanner, and Voos 2000). The driving force of protein import into mitochondria is, alongside the electric potential across its membranes, an ATP-dependent molecular motor centered around the mitochondrial HSP70 (mtHSP70) (Matouschek, Pfanner, and Voos 2000). This matrix-resident chaperone associates with the TIM complex (Schneider et al. 1994; Matouschek, Pfanner, and Voos 2000) and binds the preprotein once it emerges through the import channel (Ostermann et al. 1990). The force powering import is then exerted by a combination of preventing the substrate's retraction, similar to a brownian ratchet (Feynman, Leighton, and Sands 1963), and active pulling (Matouschek, Pfanner, and Voos 2000). Importantly, mtHSP70 can only engage a preprotein once its N-terminus emerges at the luminal side of the TIM complex (Ungermann, Neupert, and Cyr 1994; Matouschek et al. 1997). Once this engagement is established, the force exerted by mtHSP70 is sufficient to catalyze the unfolding of folded preprotein domains remaining in the cytosol, thereby rendering the entire substrate translocation competent (Matouschek et al. 1997; Matouschek, Pfanner, and Voos 2000; Matouschek 2003). It has been shown that in folded preproteins a minimum presequence length of about 50-60 amino acids is required to allow this sequence to span both membranes in the TOM and TIM complexes and engage mtHSP70, as a shorter presequence leads to inefficient import or dissociation of the folded preprotein from the outer mitochondrial membrane (Ungermann, Neupert, and Cyr 1994; Matouschek et al. 1997). However, the mitochondrial translocation machinery is unable to unfold a DHFR moiety stabilised by methotrexate (Ostermann et al. 1990; Ungermann, Neupert, and Cyr 1994; Matouschek et al. 1997). It was demonstrated that preproteins spanning both mitochondrial membranes can lead to a tight spacial association of TOM and TIM complexes called translocation contact sites (Schülke, Sepuri, and Pain 1997; Rassow et al. 1989). Intriguingly, a study showed that protein import into mitochondria is blocked when its import machinery is saturated with constructs consisting of a presequence that is long to span both membranes followed by a DHFR moiety stabilised by methotrexate (Rassow et al. 1989).

Based on the observations from our experiments with mDHFR constructs and on our knowledge of protein translocation into mitochondria, we can develop a model of protein export by *Plasmodium* parasites. If the TMD of exported *Plasmodium* proteins serves as a translocation signal for PTEX as outlined above, it would be comparable to the presequence in mitochondrial matrix proteins. The spacer length in our constructs would then have a role similar to the presequence length in mitochondrial preproteins, as it would determine whether the

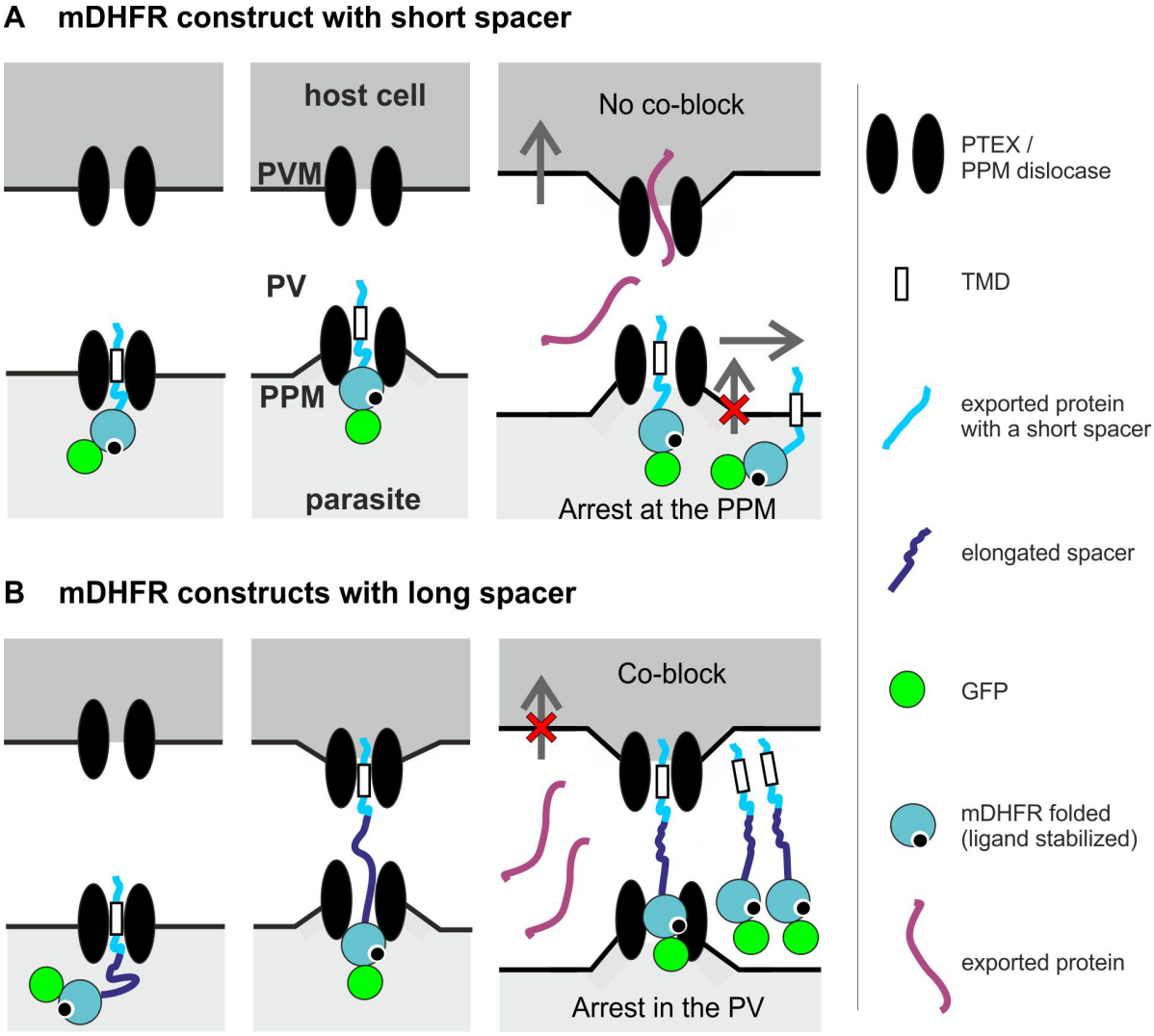

Figure (4.2): Model for co-blocking and non-co-blocking phenotypes observed with mDHFR constructs. An explanation of the symbolic representations is given to the right. **A**: A short spacer does not permit the TMD to be recognised by PTEX before the dislocation process is interrupted by the folded mDHFR moiety. The substrate is released laterally into the PPM, leading to the non-co-blocking phenotype. **B**: A long spacer permits the handover of the TMD to PTEX before the dislocation process is halted by the mDHFR moiety. The substrate jams PTEX and blocks other exported proteins from entering PTEX, leading to the co-blocking phenotype. Assuming that the co-blocking construct is dislocated from the PPM, it accumulates in the PV (see Figure 4.4).

translocation machinery at the second membrane can be engaged. In such a scenario, the co-block phenotype would correspond to mDHFR constructs with a long spacer engaging PTEX at the PVM after recognition of their TMD, while their C-terminal mDHFR moiety inhibits the complete translocation across the PPM (Figure 4.2 B). The non-co-blocking phenotype would correspond to constructs with a spacer that is too short to make contact with PTEX and thus simply remain in the PPM (Figure 4.2 A). Importantly, we determined the critical

spacer length that distinguishes the non-co-blocking from the co-blocking phenotype to lie between 34 and 99 amino acids, which is in agreement with the finding that 50-60 amino acids are needed in mitochondrial presequences to span two membranes and to engage a translocation factor in the matrix (Matouschek et al. 1997). In contrast, truncation studies carried out in our lab demonstrated that a long N-terminus preceeding the TMD does not confer co-blocking ability (Mesen-Ramirez 2016). As it was shown that co-blocking SBP1-mDHFR-GFP interacts with EXP2 (Mesen-Ramirez et al. 2016; Mesen-Ramirez 2016) and it has been proposed that PTEX could act as an integrase for PVM proteins (Haase and Koning-Ward 2010; Kalanon et al. 2016), it is plausible to hypothesise that PTEX might recognise and bind TMDs of proteins that are targeted to the PVM and beyond.

Although a recent study provided evidence that PVM integration of TM proteins is independent of HSP101 and PTEX150 (Tribensky et al. 2017), EXP2 has not been ruled out as a potential PVM integrase. While PTEX as a whole appears to be non-functional during liver stages, EXP2 is essential for parasital development in hepatocytes (Kalanon et al. 2016) (see section 1.3.2). At the same time, a mulitude of integral PVM proteins are important for the development of the parasite in the liver (Nyboer et al. 2017). Thus, EXP2 could act as a PVM integrase independent of other PTEX components in both liver and blood stages, and TMD recognition would likely be required for such a function. Additional trafficking signals of blood-stage substrates, such as a processed PEXEL N-terminus, could then lead to their translocation across the PVM with the help of the other PTEX components. The different possible functions of EXP2 may be investigated more easily once a cell line is available that can conditionally be depleted of the gene product (similar to the approaches used to investigate HSP101 and PTEX150 (Elsworth et al. 2014; Beck et al. 2014)).

The model of protein export in *Plasmodium* sketched in figure 4.2 also fits with our data obtained from experiments with BPTI constructs. Similar to co-blocking phenotypes, two phenotypes of parasites expressing BPTI constructs were observed: first, partial arrest of the constructs in the parasite periphery for soluble proteins as well as TM proteins with a short spacer; and second, unimpeded export of TM proteins with a long spacer (section 3.5.1, Figure 3.10). Due to its redox-sensitive folding, we can assume that the BPTI moiety only folds if it is exposed to the PV lumen. This clearly was the case for soluble exported BPTI constructs that are released into the PV after the fusion of secretory vesicles with the PPM. In constructs with a short spacer, the protein is dislocated entirely from the PPM before the TMD can be handed over as an export signal to PTEX, thus exposing the BPTI moiety to the PV lumen whereupon the protein folds (Figure 4.3 A). Constructs with a long spacer are handed over to PTEX and enter the second translocon without exposing the entire BPTI moiety to the PV lumen, preventing the formation of the stabilising disulfide bonds. This way the construct remains in a translocation competent state and its export is unaffected

by the BPTI moiety (Figure 4.3 B). The leakiness in the phenotype with partially arrested constructs could be due to some constructs being translocated before their BPTI moiety can fold, or to breaking of the moiety's disulfide bond by an enzyme like the PTEX-associated thioredoxin-2 (Koning-Ward et al. 2009). The latter possibility would also explain why BPTI constructs do not cause a co-block (Mesen-Ramirez 2016). Alternatively, BPTI, which in a folded state has a relatively small diameter of about 12-19 Å (Schwartz and Matouschek 1999) might be too small to completely jam the translocon. This could then also lead to partial translocation in a folded state at a slow rate, as it has been shown for some constructs with internal disulfide bridges in mitochondria (Schwartz and Matouschek 1999), which would also explain the observed leakiness of the phenotype.

Several assumptions in these models have not been experimentally addressed. First, if secretory vesicles fuse with the PPM and exported TM proteins end up spanning this membrane, it is unclear how the TMD is subsequently extracted to engage a receptor in the PV. Extraction of TM proteins out of lipid bilayers, frequently referred to as membrane dislocation, has been described in various organisms and cellular compartments. For example, FtsH is a membrane bound AAA-type protease in the bacterial plasma membrane that degrades integral membrane proteins by dislocating them towards the cytosol (Chiba et al. 2000; Akiyama and Ito 2003). Several FtsH homologues exist in chloroplasts (Rodrigues, Silva-Filho, and Cline 2011). Two AAA-type proteases with the ability to dislocate and degrade TM proteins are known to reside in the mitochondrial inner membrane with their catalytic domains exposed to opposite surfaces: that of i-AAA towards the intermembrane space and that of m-AAA towards the matrix (Leonhard et al. 2000; Gerdes, Tatsuta, and Langer 2012). Msp1/ATAD1 is a membrane-bound AAA+ ATPase localising to the cytosolic surfaces of peroxisomes and mitochondria and acting as a dislocase for tail-anchored proteins, releasing its substrates into the cytosol (Chen et al. 2014; Okreglak and Walter 2014; Weir et al. 2017; Wohlever et al. 2017). Dislocation (or retrotranslocation) of TM proteins also occurs in the ER-associated degradation pathway (ERAD), but despite a considerable body of research on this topic it remains an open question which proteins associated with ERAD actually form the channel to extract substrates out of the membrane. However, the cytosolic AAA+ ATPase CdC48/p97 is considered to be the driving motor of retrotranslocation in ERAD (Brodsky 2012; Ruggiano, Foresti, and Carvalho 2014; Avci and Lemberg 2018). Thus, dislocation of TM proteins is a common cellular mechanism driven by hexameric AAA ATPases. It suggests itself to hypothesise that HSP101 as a member of this protein family could provide the energy for the dislocation of proteins from the PPM into the PV lumen where they can be passed on to the PTEX translocon. Fitting with our model in which the folded mDHFR moiety impedes substrate extraction from the PPM, dislocation by FtsH and the mitochondrial AAA proteases is dependent on substrate unfolding (Leonhard et al.

2000; Akiyama and Ito 2003).

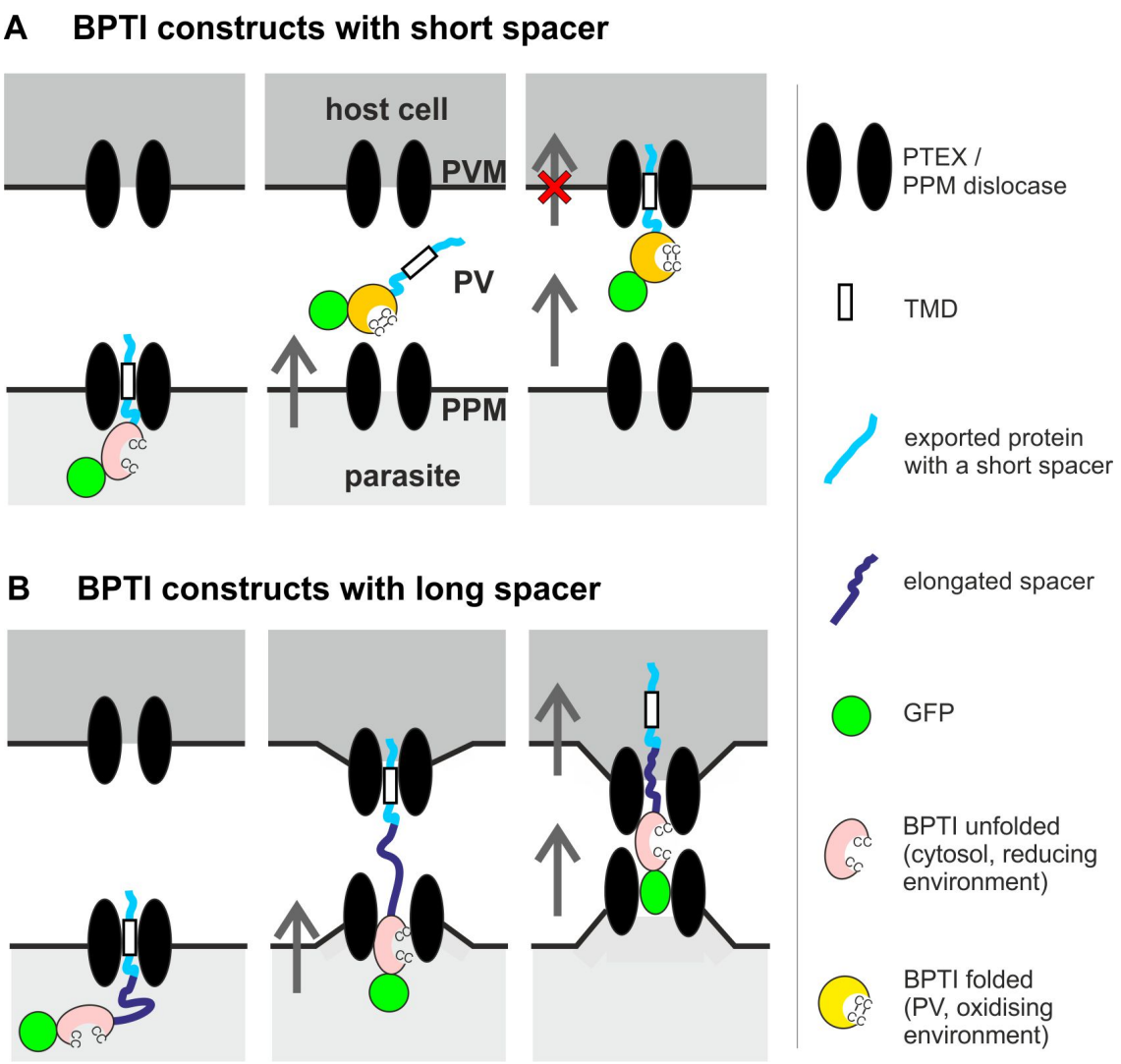

Figure (4.3): Model for the observed export phenotypes of BPTI constructs. An explanation of the symbolic representations is given to the right. **A**: A construct with a short spacer must be dislocated sufficiently from the PPM before it can engage PTEX. Once the BPTI moiety is exposed to the oxidising environment of the PV lumen, it folds and inhibits translocation across the PVM, leading to the partially arrested phenotype. **B**: A long spacer permits the engagement of the TMD with PTEX before the dislocation process is completed, while the BPTI moiety is still located on the cytosolic side of the PPM. The substrate is then handed over directly from the dislocase to PTEX without exposing the BPTI moiety to the PV lumen. The construct remains in an unfolded and thus translocation competent conformation and is exported into the RBC cytosol, leading to a phenotype with unimpeded trafficking of the construct.

This consideration gives rise to a second question: if the extraction of a non co-blocking TM protein out of the PPM is blocked by a folded C-terminal mDHFR moiety, why is the dislocase machinery not jammed by those arrested constructs - in other words, why is there

no co-block of TM proteins observed at the PPM? A possible explanation could be that substrates enter a PPM-dislocase laterally, as has been proposed for FtsH and mitochondrial AAA peptidases (Lee et al. 2011), and are analogically released into the PPM as the extraction process is inhibited by the closely folded domain. Substrate release triggered by tightly folded protein domains is known from bacterial AAA ATPases (Olivares, Baker, and Sauer 2016). Lateral release of TM proteins out of protein conducting channels has been described for bacterial translocons in the plasma membrane and the sec61 translocon in the ER (Cymer, Heijne, and White 2015) as well as for the mitochondrial TOM complex (Harner, Neupert, and Deponte 2011) and thus appears to be a common cellular mechanism. This would allow other TM proteins to be dislocated from the PPM into the PV and handed over to PTEX while arrested non co-blocking constructs accumulate in the PPM (Figure 4.2). Lateral release of these constructs would also explain the smooth distribution of fluorescence observed in arrested non-co-blocking constructs such as REX2-mDHFR-GFP and PTP1-mDHFR-GFP. This phenotype may be a correlate of constructs dispersed evenly throughout the PPM. In constrast, the "rough" fluorescence pattern with protrusions which was observed with co-blocking constructs could be a result of protein aggregations in the PV, possibly around previously proposed export sites (Bullen et al. 2012; Matz et al. 2015b).

So far, our model of protein export would suggest that a co-block occurs when the TMD that serves as an export signal is dislocated from the PPM and handed over to PTEX, initiating the translocation process at the PVM, until the folded mDHFR moiety reaches the PPM and the export process grinds to a halt. Thus, a co-blocking construct would end up spanning at least the PPM and possibly the PVM, protruding into the RBC cytosol if the N-terminus was sufficiently long. However, proteinase protection assays carried out in our lab seem to challenge this notion (Mesen-Ramirez et al. 2016; Mesen-Ramirez 2016). For these proteinase K (PK) experiments, parasites expressing SBP1-mDHFR-GFP with an additional proteinase-sensitive C-terminal tag were grown in the presence of WR. The infected RBCs were treated with tetanolysin to permeabilise the RBC membrane and subsequently with saponin to permeabilise the PVM, consecutively rendering the corresponding compartments accessible to PK (Figure 4.4). The resulting cell fractions were probed with antibodies against mDHFR (and endogenous controls to ascertain only the correct compartments were permeabilised). No protected fragment indicative of the construct spanning either PVM or PPM was detected. The full-length construct was detected in tetanolysin-treated cells, while only protease-resistant cores (mDHFR-GFP) were detected when cells were treated with saponin, indicating that the folded C-terminus of the arrested construct resides inside the PV (Figure 4.4).

The fact that no protected fragment corresponding to the digestion of an N-terminal part protruding into the RBC cytosol was detected could result from the amount of constructs ar-

rested in the PV vastly exceeding the amount of constructs actually stuck in PTEX (spanning the PVM, Figure 4.4 A). For example, it has been estimated that just about 4200 polypeptides are sufficient to saturate the import machinery of a mitochondrion (Rassow et al. 1989). The number of full-length contructs that accumulate in the PV likely is much larger than the number of PTEX translocons (assuming that one or a few constructs are sufficient to jam one translocon), which makes the detection of N-terminally cleaved constructs by western blot difficult. It is also possible that PTEX is blocked by arrested constructs while the substrates' N-termini still remain in the PV.

The fact that the folded C-terminus of the arrested construct was accessible to protease after permeabilisation of the PVM (Figure 4.4) raises a more puzzling question: How does the folded mDHFR moiety cross the PPM? To my best knowledge, unfolding of ligand bound DHFR by a cellular process has never been described. In contrast, neither the import motor of mitochondria nor the 26S proteasome, both of which are normally able to unfold the secondary structure of their substrates, are able to unfold ligand bound DHFR (Matouschek et al. 1997; Johnston et al. 1995). Thus, it seems unlikely that mDHFR is unfolded to be transferred across PPM. Another explanation could be that the folded protein is translocated across the PPM in a folded state (Figure 4.4 A). Several examples for such a process are known. In bacteria, chloroplasts and some nonphotosynthetic plastids, the twin arginine translocation (TAT) system forms variable channels through which folded substrates with a diameter of up to 70 Å are transferred (Clark and Theg 1997; Hynds, Robinson, and Robinson 1998; Gohlke et al. 2005). While no homologues of the TAT components have been annotated in the *P. falciparum* genome, it is likely that the corresponding genes were present in the apicoplast genome at some point earlier in the parasite's evolution (Kamikawa et al. 2015). Folded and even oligomerized proteins are also translocated into peroxisomes (Häusler et al. 1996; Meinecke et al. 2010; Meinecke, Bartsch, and Wagner 2016). Likewise, this is thought to be achieved by a pore with flexible diameter (Meinecke et al. 2010). Apparently, peroxisomes were lost during the evolution of *Plasmodium* but are present in other Apicomplexa and common ancestors (Schlüter et al. 2006; Ludewig-Klingner et al. 2018). Translocation of folded proteins has furthermore been reported to occur in the ERAD pathway mentioned above (Tirosh et al. 2003) but might be limited to some substrates (Bhamidipati et al. 2005; Ruggiano, Foresti, and Carvalho 2014). Homologues of ERAD associated proteins are involved in protein import into plastids of red algal origin. This mechanism is also referred to as symbiont-derived ERAD-like machinery (SELMA) (Stork et al. 2012). SELMA components have been found to be essential for protein import into the apicoplast (Agrawal et al. 2013). Components of one of these described mechanisms may have been reassigned in *Plasmodium* to serve as a dislocase or translocon for folded substrates at the PPM. Alternatively, either a completely new mechanism may have evolved in this organism,

or PTEX may play an additional role as a PPM dislocase. It is also possible that mDHFR constructs with a long spacer are ripped through the PPM by the sheer force of the translocation machinery in a process that does not physiologically occur in wild type parasites. However, to my best knowledge this would be a process without any comparable precedent. In our model in Figure 4.2 we postulate the existence of a dislocation mechanism in the PPM that is able to translocate the folded mDHFR domain from the parasite cytosol into the PV, and a scheme for the PK protection assay according to this model is shown in Figure 4.4 A.

It would be surprising if the parasite should really possess a mechanism to pull folded proteins through the PPM while such a mechanism does not exist at the PVM. An alternative model has previously been suggested in which parasite TM proteins targeted to the host cell are trafficked to the PV in a soluble state (Deponte et al. 2012; Spielmann and Gilberger 2015). For example, if chaperones would keep a substrate in a soluble state (Figure 4.4 B) or prevent its folding before it reaches the PV, this could explain the fact that the folded C-terminal mDHFR moieties of exported constructs were found in the PV. However, direct experimental evidence for such a mechanism is lacking.

Another possibility that can not be entirely ruled out is that the tagged mDHFR construct analysed in the PK protection assay might have type II membrane topology, i.e. in the PPM its C-terminus might be oriented towards the PV lumen (Figure 4.4 C). The topogenesis of TM proteins is a complex process with a variety of determinants (Shao and Hegde 2011) and may be affected by amino acids more than 50 residues away from the TMD (Kida et al. 2006; Fujita et al. 2010). Although SBP1 is assumed to have type I topology (Saridaki et al. 2009) it is conceivable that a long C-terminal appendage like mDHFR-GFP might interfere with a construct's natural topology. The widely used bioinformatics tool TMHMM (Krogh et al. 2001) predicts that tagging SBP1 with mDHFR-GFP switches its topology from type I to type II. In such a scenario, the PPM dislocase would have to engage SBP1 independently of any N-terminal signal. The TMD could be sufficient for this engagement, as it has been shown to contain information crucial for the export process (Saridaki et al. 2009; Haase et al. 2009; Gruring et al. 2012), or the dislocase could extract PPM proteins without further substrate specificity, like it has been suggested for the mitochondrial dislocase Msp1 (Weir et al. 2017). This enzyme appears to dislocate TM proteins indescriminately unless they are protected by interaction partners (Weir et al. 2017). Again, direct evidence for exported TM proteins being trafficked in type II topology is lacking, but I suggest to take this possibility into account for the design of future experiments.

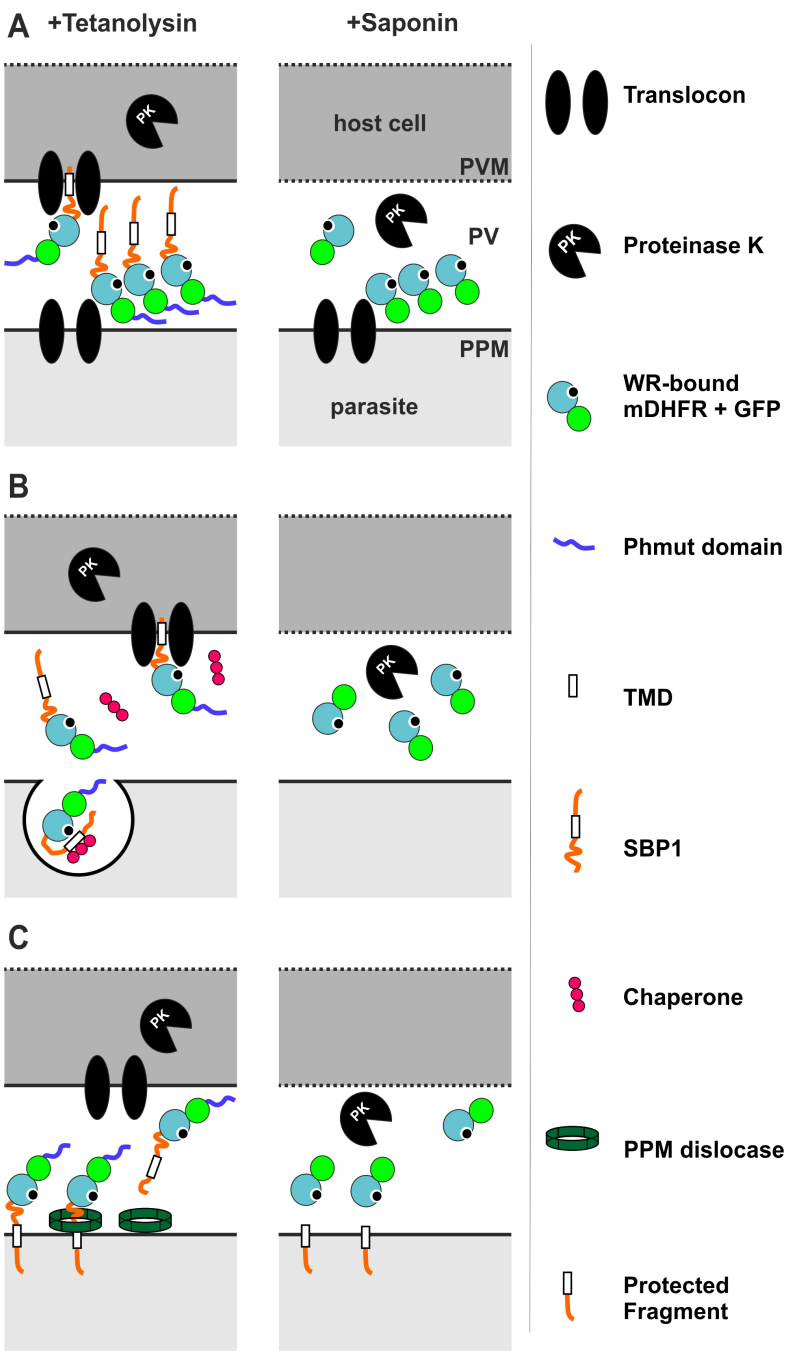

Figure (4.4): Possible scenarios leading to the detection of the folded mDHFR moiety of a co-blocking construct in the PV by a PK protection assay (Mesen-Ramirez 2016). RBCs with parasites expressing SBP1-mDHFR-GFP-PHmut and grown in the presence of WR were treated with tetanolysin to permeabilise the RBC membrane (left collumn) followed by saponin treatment to permeabilise the PVM (right collumn), consecutively rendering the corresponding compartments accessible to PK. **A**: The construct is translocated across the PPM in a folded state by a mechanism that may involve PTEX or another unknown machinery. PTEX translocation across the PVM is jammed by the stably folded construct. Large amounts of the full-length construct accumulate in the PV, exceeding the amount of N-terminally cleaved construct stuck in PTEX by far. **B**: The construct is trafficked to the PV in a chaperone-bound state. The export block is induced once the construct is passed over to PTEX. **C**: The construct is trafficked to the PV in type II topology. It is released from the PPM by a dislocase that recognises its TMD, and handed over to PTEX. Protected construct fragments in the PPM are not detected by antibodies against mDHFR.

Conclusion

We have used conditionally foldable translocation intermediates to investigate the sequence of events leading to the export of parasite proteins across PPM, PV and PVM into the host cell. We have verified a translocation event at the PVM to be a converging point in the trafficking pathways of all tested protein types. In addition, we have discovered that in exported TM proteins, the distance between their TMD and a tightly folded moiety following the TMD C-terminally plays a role for export. We interpret these data to substantiate a model in which two translocation steps (or, more precisely, a dislocation step followed by a translocation step) are required for the export of TM proteins into the host cell. Topological considerations let us propose that tightly folded translocation intermediates can jam the export machinery at the PVM, termed PTEX, leading to a breakdown of PTEX function and thus abolishes protein export. Additional research conducted by colleagues in our laboratory demonstrated that folded translocation intermediates lead to impaired parasite development similar to induced knock-down of PTEX components, and that such intermediates associate with the PTEX component EXP2, confirming our proposition.

The complex cellular processes that have evolved in the interactions of *Plasmodium* parasites with their host cell are tantalising for both their biological uniqueness and their potential targetability for antimalarial compounds. Many open questions concerning these parasite-host interactions remain. Further work will be needed to elucidate the mechanisms of protein dislocation from the PPM and insertion into the PVM, as well as the functionality of PTEX itself. Whether EXP2 can be identified as the definite protein conducting pore for exported proteins and what other pivotal role for parasite development it might play is under investigation, as well as the role of several new proteins that have been found to associate with PTEX. The trafficking and targeting of PNEPs, including the major virulence factor *Pf*EMP1, to the host cell remains to be fully understood. Furthermore, much remains to be learned about protein trafficking beyond the PVM and the interactions between parasital and erythrocytic proteins.

In the past, the biological peculiarities of *Plasmodium* parasites have posed substantial obstacles for the study of these organisms. Several recent innovations in the area of genome editing have been shown to be applicable in *Plasmodium* parasites and will hopefully contribute to expedite our understanding of the malaria pathogen and to develop new tools to combat this disease.

Zusammenfassung

Mithilfe von Translokationssubstraten mit Domänen, die unter bestimmten Bedingungen an der Entfaltung gehindert werden können, haben wir die Abfolge der Prozesse untersucht, die den Export von Proteinen über die PMP, die PV und die PVM hinweg bis in die Wirtszelle ermöglichen. Wir haben gezeigt, dass die Exportwege aller von uns untersuchten Proteintypen in einem Translokationsschritt an der PVM zusammenlaufen. Außerdem haben wir festgestellt, dass bei exportierten Transmembranproteinen der Abstand zwischen ihrer Transmembrandomäne und einer nachfolgenden, nicht entfaltbaren Domäne entscheidend für ihren Exportphänotyp ist. Nach unserer Interpretation untermauern diese Ergebnisse ein Modell, dem zufolge exportierte Transmembranproteine zunächst von der PMP dislozierd werden müssen, um danach über die PVM transloziert zu werden. Topologische Überlegungen sprechen unserer Ansicht nach dafür, dass nicht entfaltbare Translokationssubstrate den PTEX Komplex (die Exportmaschinerie an der PVM) verstopfen können, was dazu führt, dass der Proteinexport durch PTEX zum Erliegen kommt. Dieses Modell wird durch weitere Experimente gestützt, die von Kollegen in unserem Labor durchgeführt wurden. So konnte gezeigt werden, dass nicht entfaltbare Translokationssubstrate die Entwicklung der Parasiten deutlich beeinträchtigen. Dieser Phänotyp ähnelt dem induzierten Knock-Down von PTEX Komponenten. Weiterhin wurde gezeigt, dass nicht entfaltbare Translokationssubstrate mit der PTEX Komponente EXP2 interagieren.

Die komplexen zellulären Prozesse, die sich aus der Interaktion von *Plasmodium* Parasiten mit ihrer Wirtszelle entwickelt haben, sind ein faszinierender Forschungsgegenstand - zum einen aufgrund ihrer biologischen Einzigartigkeit, zum anderen, da sie potenzielle Ziele neuer Wirkstoffe gegen Malaria darstellen. Nach wie vor existieren zahlreiche offene Fragen rund um diese Interaktionen zwischen Parasit und Wirt. Weitere Anstrengungen werden etwa nötig sein, um die Dislokation von Proteinen aus der PMP und ihre Insertion in die PVM zu verstehen und die Funktionsweise von PTEX aufzuklären. Es wird gegenwärtig untersucht, ob EXP2 tatsächlich die Pore des Translokationskanals bildet, und welche weiteren Funktionen es in der Entwicklung des Parasiten erfüllt. Auch eine Reihe weiterer Proteine, die in Zusammenhang mit PTEX gebracht wurden, werden derzeit untersucht. Der Transport von PNEPs einschließlich des Hauptvirulenzfaktors *Pf*EMP1 in die Wirtszelle ist ebenfalls noch nicht vollständig verstanden. Schließlich bedürfen auch der Transport von Parasitenproteinen durch das Zytosol des Erythrozyten und ihre Interaktionen mit Proteinen der Wirtszelle weiterer Forschung, um unser Verständnis des Exportprozesses zu vervollständigen.

In der Vergangenheit haben die biologischen Besonderheiten von *Plasmodium* Parasiten die Forschung an diesen Organismen deutlich erschwert. Eine Reihe jüngerer Innovationen auf

dem Gebiet des Genome Editing könnten nun dazu beitragen, die Erforschung des Malariaerregers zu beschleunigen, und so dabei helfen, neue Werkzeuge zu entwickeln, um diese Krankheit zu besiegen.

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Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....

Ferdinand Reinsch