Characterization of two *Plasmodium falciparum* Proteins, MAHRP1 and MAHRP2, Involved in Host Cell Refurbishment

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Summary

Malaria is one of the leading causes of morbidity and death in the world. Responsible for the most virulent form of the disease is the *Apicomplexan* parasite *Plasmodium falciparum* transmitted by the female *Anopheline* mosquito. Today, no vaccine is commercially available. Eradication of malaria failed due to the evolution of drug resistance in the parasite and insecticide resistance in its mosquito vector but is since 2007 again on the agenda of health officials. The understanding of the biology of *P. falciparum* is limited impeding the identification of new intervention targets.

Disease and death is triggered by blood stages where the parasite undergoes multiple rounds of replication. During this part of the life cycle *P. falciparum* lives surrounded by a parasitophorous vacuole in the terminally differentiated red blood cell which is metabolically highly reduced lacking compartments, a nucleus, and a protein trafficking machinery. Nutrient supply is also limited. To survive in such an environment the parasite needs to refurbish its host cell inducing remarkable modifications such as the formation of membranous structures termed Maurer's clefts in the cytosol of the erythrocyte, and knobs on the surface of the red blood cell. The refurbishment processes are initiated by the export of parasite derived proteins beyond the confines of its own plasma membrane, across the parasitophorous vacuolar membrane into the cytosol of the erythrocyte or to the erythrocyte membrane. The major virulence factor *Pf*EMP1 is exported to knobs where it binds as surface exposed molecule to endothelial receptors thereby mediating cytoadherence and sequestration of mature-stage infected erythrocytes in blood capillaries evading clearance by the spleen. This is the key process accounting for clinical symptoms of malaria such as organ failure or cerebral malaria.

Not much is known on how parasite proteins are secreted along such a complex route crossing several membranes. Proteins are thought to be classically secreted into the parasitophorous vacuole. A *Plasmodium* export element (PEXEL) has been identified in most exported proteins which is recognized by a translocon in

the parasitophorous vacuolar membrane enabling secretion into the erythrocyte cytosol. As an exception to the rule, there are a number of proteins described being exported but lacking such a motif.

In this thesis, we focus on the processes involved in host cell refurbishment as well as on the export mechanism of two of those PEXEL-negative proteins. The aim was to characterize two proteins termed Membrane Associated Histidine-Rich Proteins 1 and 2 (MAHRP1 and MAHRP2) which are exclusively transcribed early during blood stage development when such refurbishment occurs. Both proteins are similar in structure carrying centrally a predicted transmembrane domain. The C-terminal domain of MAHRP1 comprises histidine-rich DHGH repeats while the N-terminal domain of MAHRP2 is histidine-rich. MAHRP1 has previously been shown to localize to Maurer's clefts whereas nothing was known about MAHRP2.

We generated parasite lines in which the *mahrp1* gene was disrupted to investigate possible functions of MAHRP1 in these knock out parasites. In erythrocytes infected with MAHRP1-deficient parasites the major virulence factor *Pf*EMP1 was not exported anymore to the surface of the erythrocyte. It was still produced but was trapped within the confines of the parasite. This resulted in a reduced ability of the infected erythrocytes to bind to the endothelial receptor CD36. The phenotype could be restored by the complementation of the MAHRP1-deficient parasites with episomal expression of the gene. These findings indicate an essential function for MAHRP1 and Maurer's clefts in the export of the major virulence factor to the surface of the red blood cell.

By immunofluorescence assays and electron microscopy as well as through transfection technology we could show that MAHRP2 is exported to recently described new structures in the infected erythrocyte, called tethers. MAHRP2 is the first and only protein so far specifically localizing to these tubular structures thought to attach Maurer's clefts to the erythrocyte membrane. Life cell imaging of infected erythrocytes expressing MAHRP2-GFP revealed both mobile and fixed populations of these structures which allowed enrichment by differential centrifugation. Solubilization studies showed that MAHRP2, although having a predicted transmembrane domain, only peripherally associates with membranes

whereas MAHRP1 represents an integral membrane protein. We failed to delete the *mahrp2* gene in several attempts indicating an essential function for MAHRP2 in parasite survival. Tagging the *mahrp2* gene with the FKBP destabilizing domain, however, resulted in a nearly complete loss of MAHRP2 protein, although with no obvious altered phenotype.

Through pull down experiments and mass spectrometric analyses of the enriched tether fraction obtained by differential centrifugation, we found several potential protein interaction partners of MAHRP2, which subsequently were GFP- or HA-tagged and transfected into parasites for further analyses.

Both MAHRP1 and MAHRP2 are exported despite lacking a classical signal sequence or a PEXEL motif. Trafficking of MAHRP1 and MAHRP2 was ER dependent. Sequences required for export of MAHRP2 were determined using transfectants expressing truncated MAHRP2 fragments. Interestingly, sequence requirements were different from MAHRP1 suggesting alternative export mechanisms. The first 15 amino acids of MAHRP2, the histidine-rich N-terminal region, and the predicted central hydrophobic region were necessary for correct trafficking. Although MAHRP2 is not an integral membrane protein, membrane association seemed to be absolutely essential for the export of MAHRP2.

A better understanding of the function of tethers and Maurer's clefts which are organelles unique to the most virulent malaria parasite *P. falciparum* as well as delineation of the export mechanisms of proteins destined for these structures should lead to the development of novel intervention strategies.

Zusammenfassung

Malaria gilt als eine der drei Infektionskrankheiten mit den höchsten Todesraten weltweit. Hierfür verantwortlich ist fast ausschliesslich die virulenteste Spezies *Plasmodium falciparum*, ein Parasit des Phylums *Apicomplexa*, der durch die weibliche *Anopheles* Mücke übertragen wird. Bis heute existiert kein zugelassener Impfstoff. Malaria auszurotten scheiterte bisher an Resistenzentwicklungen sowohl des Parasiten gegen Antimalaria-Medikamente wie auch der Vektormücke gegen Insektizide. Seit 2007 ist die Malaria-Eradikation wieder auf dem Programm von Gesundheitsorganisationen. Das biologische Verständnis von *P. falciparum* ist allerdings noch stark lückenhaft, aber das Verständnis von kritischen Prozessen im Lebenszyklus des Parasiten könnte die Identifikation neuer und innovativer Interventionsstrategien ermöglichen.

Krankheit und Tod durch Malaria wird ausschliesslich von Blutstadien des Parasiten verursacht, in welchen er vielfache Replikationen durchführt. Während dieses Stadiums des Lebenszyklus ist *P. falciparum* von einer parasitophoren Vakuole in der terminal differenzierten roten Blutzelle umgeben. Diese ist metabolisch stark reduziert, ohne Kompartimentierung, ohne Kern, ohne Transportmaschinerie für Proteine, und der Zugriff auf Nährstoffe ist ebenfalls limitiert. Um in einer solchen Umgebung überleben zu können, muss der Parasit seine Wirtszelle neu einrichten, was wiederum zu erheblichen Modifizierungen führt. Der Parasit exportiert Membranstrukturen, sogenannte Maurer'sche Spalten, in das Zytosol der roten Blutzelle und knubbelartige Strukturen auf die Oberfläche der Wirtszelle. Diese Modifikationen benötigen den Export von Parasitenproteinen durch die parasitäre Plasmamembran hindurch und durch die parasitophore Vakuolenmembran ins Zytosol oder an die Oberfläche der roten Blutzelle. Der bedeutendste Virulenzfaktor *Pf*EMP1 wird in der Erythrozytenmembran eingelagert, wo er als oberflächenexponiertes Molekül an Rezeptoren des Kapillaren-Endothels bindet und so Zytoadhärenz und Sequestration von infizierten roten Blutzellen herbeiführt. Somit weicht der Parasit einerseits einer Aussonderung durch die Milz aus, andererseits ist aber genau dieser Prozess hauptverantwortlich für die klinischen Symptome von

Malaria und verantwortlich für die Mortalität dieser Krankheit z. B. durch Organversagen oder zerebrale Malaria.

Gern wüsste man mehr darüber, wie die erwähnten Parasitenproteine entlang eines so komplexen Weges mit diversen Membranbarrieren sekretiert werden. Man nimmt an, dass die Sekretion exportierter Proteine in die parasitophore Vakuole auf herkömmliche Weise erfolgt. Für Proteine, die jedoch darüber hinaus exportiert werden sollen, ist der Mechanismus weniger klar, aber die meisten dieser exportierten Proteine besitzen ein ‚*Plasmodium*-Export-Element' (PEXEL), welches von einem Translokon in der parasitophoren Vakuolenmembran erkannt wird und so die Sekretion ins Zytosol des Erythrozyten ermöglicht. Es sei hier angemerkt, dass es jedoch eine Anzahl von Proteinen gibt, die exportiert werden, obwohl ihnen ein solches Motiv fehlt.

Die vorliegende Arbeit ist einerseits auf die Prozesse fokussiert, die die Wirtszelle modifizieren, andererseits konzentriert sie sich aber auch auf den Exportmechanismus von zwei PEXEL-negativen Proteinen. Die beiden Proteine MAHRP1 und MAHRP2 (Membrane Associated Histidine-Rich Proteins 1 and 2), welche exklusiv sehr früh transkribiert werden, nämlich während des Blutstadiums, in dem der Umbau der Wirtszelle erfolgt, konnten charakterisiert werden. Diese Proteine sind reich an Histidinen und strukturell ähnlich mit einer zentralen, prognostizierten Transmembrandomäne. Die C-terminale Domäne von MAHRP1 beinhaltet histidinreiche DHGH-Repeats, während MAHRP2 eine histidinreiche N-terminale Domäne aufweist. Frühere Studien haben gezeigt, dass MAHRP1 an den Maurer'schen Spalten lokalisiert ist. Die Lokalisation von MAHRP2 hingegen war bisher unbekannt.

Zur genaueren Analyse wurden Parasitenlinien generiert, in denen das *mahrp1* Gen eliminiert wurde, um hiermit mögliche Funktionen von MAHRP1 zu untersuchen. In Erythrozyten, welche mit *mahrp1*-defizienten Parasiten infiziert waren, konnte gezeigt werden, dass der Virulenzfaktor *Pf*EMP1 nicht mehr an die Oberfläche des Erythrozyten transportiert wurde. Es wurde festgestellt, dass *Pf*EMP1 zwar noch produziert wurde, aber dass kein Export über die Grenzen des Parasiten hinaus mehr statt fand. Die Bindung an den Endothelrezeptor CD36 war bei diesen

infizierten Erythrozyten stark reduziert. Durch Komplementierung der *mahrp1*-defizienten Parasiten mit episomal exprimiertem MAHRP1 konnte dieser mangelhafte Phänotyp wieder aufgehoben werden. Diese Befunde deuten auf eine essentielle Funktion von MAHRP1 und der Maurer'schen Spalten im Export von *Pf*EMP1 zur Oberfläche der roten Blutzelle hin.

Zu Beginn dieser Arbeit existierte keine Information über MAHRP2, aber durch Immunofluoreszenz- und Elektronenmikroskopie wie auch mittels Transfektions-technologie konnte gezeigt werden, dass MAHRP2 zu neuen, erst kürzlich beschriebenen Strukturen im infizierten Erythrozyten exportiert wird. Diese neuen Strukturen werden als ‚Tethers' bezeichnet, und MAHRP2 ist bisher das erste und einzige Protein, das sich spezifisch an diese tubulären Strukturen lagert, von denen angenommen wird, dass sie die Maurer'schen Spalten an die Erythrozytenmembran anheften. Zeitrafferphotographie von infizierten Erythrozyten, die das Fusionsprotein MAHRP2-GFP exprimierten, liessen sowohl mobile, wie auch fixierte Populationen von ‚Tethers' erkennen, was eine Anreicherung durch differentielle Zentrifugation erlaubte. Löslichkeitsstudien ergaben, dass MAHRP2 trotz einer prognostizierten Transmembrandomäne nur peripher mit Membranen assoziiert ist, während MAHRP1 ein integrales Membranprotein repräsentiert. Versuche, das *mahrp2* Gen zu deletieren, scheiterten mehrfach, was auf eine für den Parasiten lebensnotwendige, essentielle Funktion von MAHRP2 hindeutet. Die Fusion des *mahrp2* Gens mit einer destabilisierenden FKBP-Domäne führte jedoch zu einem beinahe kompletten Verlust von MAHRP2 Protein, wenn auch nicht zu einem offensichtlich verändertem Phänotyp.

‚Pull Down'-Experimente und massenspektrometrische Analysen der durch differentielle Zentrifugation angereicherten ‚Tether'-Fraktion erlaubten die Identifikation diverser potentieller Protein-Interaktionspartner von MAHRP2. Diese Proteine wurden anschliessend für weitere Analysen als GFP- oder HA-markierte Proteine in Parasiten transfiziert.

In dieser Arbeit konnte gezeigt werden, dass MAHRP1 und MAHRP2 trotz fehlender klassischer Signalsequenz oder fehlendem PEXEL-Motiv in das Zytosol

des Erythrozyten exportiert werden. Der Transport von MAHRP1 und MAHRP2 war ER abhängig, und die benötigten Sequenzen für den Export von MAHRP2 wurden unter Verwendung von transfizierten Parasiten ermittelt, die verkürzte Fragmente von MAHRP2 exprimierten. Interessanterweise unterschieden sich die für den Export benötigten Sequenzen von denen von MAHRP1, was auf unterschiedliche Exportmechanismen für die beiden Proteine hinweist. Die ersten 15 Aminosäuren von MAHRP2, die histidinreiche N-terminale Region, sowie die zentrale hydrophobe Region waren für den korrekten Transport nötig. Obwohl MAHRP2 sich nicht wie ein integrales Membranprotein verhält, scheint eine Membranassoziation für den Export von MAHRP2 absolut essentiell.

‚Tethers' sowie Maurer'sche Spalten sind Organelle, die scheinbar ausschliesslich im virulentesten Malariaparasiten *P. falciparum* vorkommen. Ein Verständnis der Funktion von ‚Tethers' sowie der Maurer'schen Spalten, wie auch die Entschlüsselung der Exportmechanismen von nicht PEXEL-tragenden Proteinen eröffnen die reelle Möglichkeit eines Beitrages zur Entwicklung von neuen Interventionsstrategien.

Abbreviations

ATS	acidic terminal sequence
BSA	bovine serum albumin
BSD	blasticidin S deaminase
DHFR	dehydrofolate reductase
EM	electron microscopy
ER	endoplasmic reticulum
ETRAMP	Early Transcribed Membrane Protein
FKBP	FK506 binding protein
GFP	Green Fluorescent Protein
HPI	hours post infection
HRP	Histidine-Rich Protein
KAHRP	Knob Associated Histidine-Rich Protein
MAHRP	Membrane Associated Histidine-Rich Protein
MC	Maurer's cleft
NPP	new permeability pathways
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEXEL	*Plasmodium* export element
*Pf*EMP1, 3	*Plasmodium falciparum* Erythrocyte Membrane Protein 1, 3
PM	parasite membrane
PNEP	PEXEL-negative protein
PTEX	*Plasmodium* translocon of exported proteins
PV	parasitophorous vacuole
PVM	parasitophorous vacuolar membrane
RBC	red blood cell
REX1,2	Ring Exported Protein 1, 2
SBP1	Skeleton Binding Protein 1
SDS	sodiumdodecylsulfate
TVN	tubulovesicular network
VTS	vacuolar transport signal

Chapter 1

Introduction:

Malaria and the Cell Biology of *Plasmodium falciparum*

Introduction

Malaria

The disease malaria is caused by the *Apicomplexan* parasite *Plasmodium*. The protozoan parasite which is transmitted by the *Anopheles* mosquito can infect humans, primates, monkeys or rodents. Mainly four species of *Plasmodium* infect humans: *P. falciparum, P.vivax, P. ovale,* and *P. malariae*. However, current observations show increased transmission of non-human primate malaria parasites such as *P. knowlesi* to humans (Putaporntip *et al.*, 2009). *P. falciparum* causes most disease and death across Sub-Saharan Africa, whilst *P. vivax* is the most prevalent parasite in most other malaria-endemic areas of the world as South-East Asia. Over two hundred million malaria cases were reported for 2008 worldwide and malaria accounted 863'000 deaths of which mainly pregnant women and children under the age of 5 were affected (World Health Organization, 2009). *P. falciparum* is by far the major cause of disease burden as it is responsible for the most virulent form of human malaria. *P. falciparum* is a descendent of the chimpanzee parasite *P. reichenowi* – all other four *Plasmodia* capable of infecting humans are derived from monkey parasites (Rich *et al.*, 2009). Clinical manifestations of severe malaria include cerebral malaria, severe anaemia, hypoglycaemia, renal failure, non-cardiac pulmonary oedema, and respiratory failure (World Health Organization, 2009). Responsible for most of these clinical symptoms is the asexual reproduction of the parasite within the red blood cell. The malaria parasite is one of only few pathogens using the red blood cell as a host cell. Two other *Apicomplexan* parasites *Theileria* and *Babesia* and two bacteria *Anaplasma* and *Bartonella* are also known to parasitize red blood cells.

Problems with malaria control exist because of the parasite's remarkable capacity for adaptation to its highly heterogeneous natural environment. The parasite adapts to two different hosts, undergoes ten morphological transitions in five different host tissues, proliferates asexually within three of these, and sexually at each transfer between hosts. In the last few years, a decrease in malaria cases has been observed due to expanded control programs improving access to preventive

and treatment measures, such as insecticide-treated bed nets (ITNs), intermittent preventive treatment of pregnant women and infants (IPTp and IPTi) and artemisinin-based combination drug therapy (World Health Organization, 2009). Today, the most advanced experimental vaccine which is being developed, RTS,S/AS, shows rather limited protection. The efficacy in infants against first infection with *P. falciparum* malaria during a 6-month period after the third vaccination is 65% (Abdulla *et al.*, 2008).

Past failures to eradicate malaria were hampered by the evolution of drug resistance in the parasite and insecticide resistance in its mosquito vector (Rieckmann, 2006). Also, animal reservoirs (monkeys, *P. knowlesi*) for human malaria will make human infection control more difficult (Prugnolle *et al.*, 2010). The understanding of the biology of *P. falciparum* remains far from complete and access to malaria-control measures remains well below the coverage required for adequate disease control. Therefore, research on asexual blood stages and the development of new therapeutics remain essential as this phase of the life cycle causes disease and death.

Recent genomic research revealed how *P. falciparum* is uniquely adapted to its host as many of its genes have no homologues in other species (Gardner *et al.*, 2002). In addition, it has a highly specialized machinery for interacting with the environment of the host (Maier *et al.*, 2009). Current research mainly focuses on the clinically most important malaria parasite, *P. falciparum*. A big advantage is the availability of an *in vitro* culturing system (Trager & Jenson, 1978) and the sequenced genome of the culture strain 3D7 and others (www.plasmodb.org).

Plasmodium falciparum life cycle

Plasmodium falciparum alternates between the human and female mosquito host of the genus *Anopheles* maintaining a complex life cycle (Figure 1). Taking a blood meal the infected *Anopheles* mosquito injects haploid sporozoites into the human host. Once in the blood stream, sporozoites migrate to the liver where they invade hepatocytes and multiply asexually. After five to twenty days, in the case of *P. falciparum* thousands of merozoites are released into the blood stream. These now invade a different type of host cell, the red blood cell, where they undergo a 48 hours cycle of asexual replication. Surrounded by a parasitophorous vacuolar membrane (PVM), the parasite develops from ring stage (0-22 hours post invasion (hpi)) to trophozoite (22-36 hpi) and finally schizont stage (36-48 hpi). Upon rupture of erythrocytes infected with schizonts up to 32 new merozoites are released into the bloodstream, which initiate a new round of schizogony.

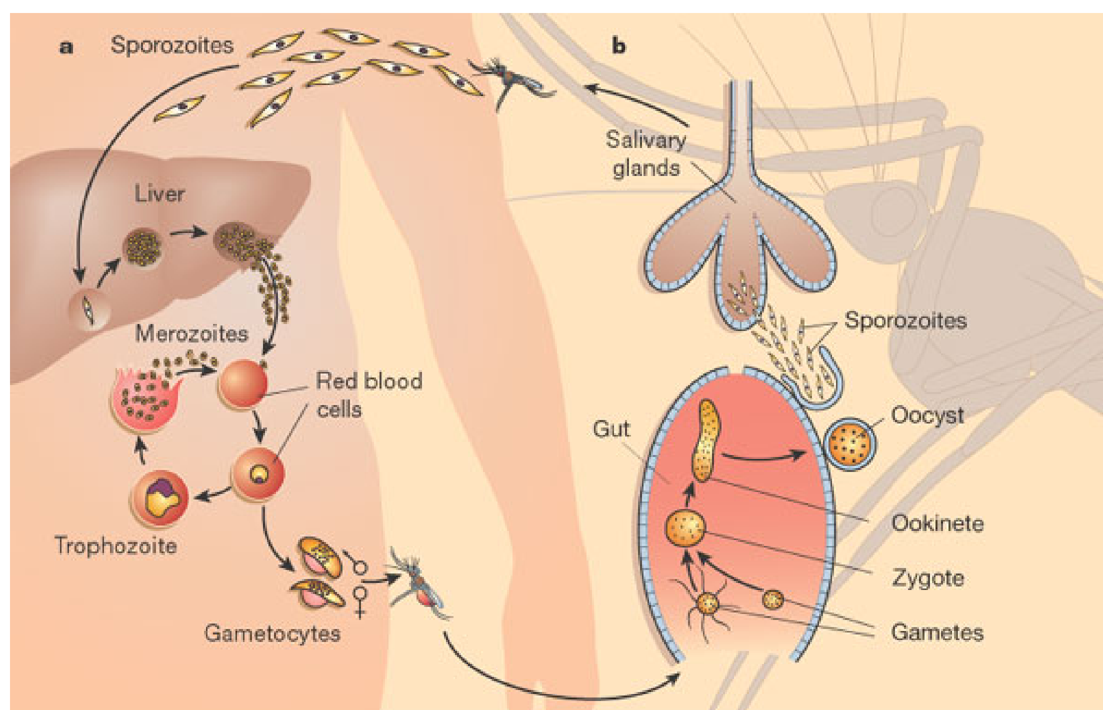

Figure 1: *P. falciparum* life cycle. (Wirth 2002, Nature 419, 495-496)

Some of the intraerythrocytic parasites develop into sexual blood stages termed gametocytes. Gametocytes must be taken up by an *Anopheline* mosquito during another blood meal. Once in the mosquito gut, gametocytes develop into gametes and sexual reproduction takes place whereby 8 flagellated

microgametes are released from a male gametocyte. The exflagellated microgamete fertilizes a female macrogamete to form a diploid zygote which further develops into an ookinete capable of penetrating the gut wall and maturing to an oocyst in about ten days. The oocyst finally ruptures releasing a large number of haploid sporozoites into the haemolymph. The sporozoites then migrate to the salivary gland from where they are injected into the human host during the next blood meal completing the cycle. The intraerythrocytic cycle where the parasite replicates asexually is responsible for the pathology of the disease, thus, our research focuses mainly on the molecular aspects of this specific part of the *P. falciparum* life cycle.

Plasmodium falciparum cell biology

P. falciparum belongs to the phylum of the *Apicomplexa* (Levine, 1985). Other members of this group of intracellular parasites include *Toxoplasma gondii* and *Cryptosporidium* which infect humans and cause disease in immunocompromised individuals, as well as parasites of domesticated animals, *Theileria, Eimeria, Neospora,* and *Babesia*. Even though there are distinct differences in the choice of the host cell, several structural features are shared between the different *Apicomplexan* parasites, mainly, the apical end invasion-related organelles called rhoptries, micronemes, and dense granules. Most *Apicomplexans* also have a rudimentary plastid, the apicoplast.

Micronemes, rhoptries, and dense granules are specialized secretory organelles densely packed with proteins. Upon contact of the merozoite with the host cell surface the small cigar-shaped electron-dense **micronemes** trigger calcium ion release and the discharge of their content which in turn mediates parasite attachment (de Souza *et al.*, 2008, Kats *et al.*, 2008). After adhesion, the long (around 400 nm) pear-shaped **rhoptries** secrete their protein content. Rhoptries have a posterior electron-dense bulb and an electron-lucent neck. The released proteins and proteases are involved in formation of the moving junction and the parasitophorous vacuole (PV) and are also internalized to form part of the PV membrane (Bannister, 2001, Bradley & Sibley, 2007, de Souza *et al.*, 2008, Kats *et*

al., 2008, Proellocks *et al.*, 2010). **Dense granules** are spherical organelles with a diameter of 0.2 µm not really part of the apical complex but rather distributed throughout the cell. The release of their protein contents into the PV takes place after parasite invasion by fusion of the dense granule membrane with the parasite plasma membrane (de Souza *et al.*, 2008, Kats *et al.*, 2008).

The **apicoplast** is a plastid surrounded by four membranes which was acquired secondarily through endosymbiosis between a free living ancestor of the *Apicomplexa* and a red alga. *Cryptosporidium* parasites are an exception due to secondary loss of this organelle (Zhu *et al.*, 2000). The apicoplast of most *Apicomplexa* no longer has any photosynthetic properties, except for the coral symbiont *Chromera velia*, supporting the hypothesis that an ancestor of the apicoplast of *Apicomplexa* was also photosyntethic (Moore *et al.*, 2008). The apicoplast is essential for the survival of *P. falciparum* (Goodman & McFadden, 2007, Vaughan *et al.*, 2009, Yu *et al.*, 2008). Several biochemical pathways such as biosynthesis of fatty acids are carried out by the apicoplast (Ralph *et al.*, 2004).

The *P. falciparum* **mitochondrion** is closely associated with the apicoplast in merozoite and ring stages. The two organelles then undergo elongation and branching as the parasite matures retaining points of association until the organelles divide and segregate into daughter merozoites as a pair (Slomianny & Prensier, 1986, van Dooren *et al.*, 2005).

A perinuclear ring with two small protrusions representing the **ER** in early stages develops into an extended reticular network during growth of the parasite (Tilley *et al.*, 2007, van Dooren *et al.*, 2005). The **Golgi** apparatus has not been observed as stacked cisternae such as in other eukaryotes. The *cis*-Golgi is seen as a perinuclear structure in early stages separated from the *trans*-Golgi compartment and later on two horns are formed close to the ER protrusions (Struck *et al.*, 2005). Several eukaryotic ER and Golgi homologues have been found such as the COPII proteins Sar1p and Sec31p or Golgi marker proteins ERD2, Rab6 or GRASP (Struck *et al.*, 2005). However, little is known about the molecular organization of the secretory pathway of *P. falciparum*.

Upon invasion the parasite digests host cell derived haemoglobin which is the main source for amino acids. Engulfing of haemoglobin involves endocytic structures, termed cytostomes, at the parasite surface, that allow direct contact of the parasite with the erythrocyte cytoplasm. (Abu Bakar *et al.*, 2010, Elliott *et al.*, 2008, Slomianny, 1990). The haemoglobin is transported to an acidic digestive vacuole, a lysosome-like compartment, the **food vacuole** (Abu Bakar *et al.*, 2010, Slomianny, 1990). Haemoglobin is digested by proteases and oxidized to hematin, a toxic molecule that can catalyze oxidative damage to lipids and proteins (Balla *et al.*, 2007, Becker *et al.*, 2004, Kumar & Bandyopadhyay, 2005). Hematin undergoes crystallization to form the less toxic malaria pigment hemozoin which is stored in the food vacuole (Slater, 1992). Haemoglobin does not contain the amino acid isoleucine, additionally, glutamate, methionine, cysteine, and proline are underrepresented (Kirk, 2001, Martin & Kirk, 2007). These amino acids, as well as other nutrients, need to be taken up from the extra cellular space.

Refurbishment of the red blood cell

The human erythrocyte is simply said a 'sack' filled with haemoglobin with a specialized task to transport O_2 and CO_2. The cell is devoid of all major histocompatibility complex (MHC) molecules allowing the parasite to hide from the immune system. The terminally differentiated cell is metabolically highly reduced. It lacks compartments and lost the nucleus and thus the genetic program to newly synthesize proteins or lipids. It is an unusual choice as host cell because nutrient supply is limited; furthermore the cell does not have a protein trafficking machinery. Consequently, the parasite induces striking modifications to the erythrocyte enabling its growth and multiplication. Novel membrane systems and compartments are formed, the permeability of the erythrocyte membrane is modified, and the infected host cell becomes adhesive to the vascular endothelium. All of this is initiated right upon invasion during the ring stage by the export of parasite derived proteins beyond the confines of its own plasma membrane, across the parasitophorous vacuolar membrane into the cytosol of the erythrocyte or to the erythrocyte membrane.

Extensions and whorls arising from the PV membrane form the **tubulovesicular network** (TVN) (Aikawa, 1971, Bannister *et al.*, 2004, Kriek *et al.*, 2003). Only very little is understood about the biogenesis of the TVN. It was shown that the Golgi marker sphingomyelin synthase is exported to the TVN indicating that it might have secretory characteristics (Elmendorf & Haldar, 1994). Inhibiting the enzyme activity blocked formation of TVN tubules (Lauer *et al.*, 1995, Lauer *et al.*, 1997). These tubules are thought to be involved in nutrient import (Lauer *et al.*, 1997). Recently, another protein was described being essential for TVN mediated nutrient import, the Erythrocyte Vesicle Protein 1 (EVP1) (Tamez *et al.*, 2008).

The adhesive phenotype of erythrocytes infected with mature parasites is associated with knobby protrusions underneath the erythrocyte plasma membrane. These **knobs** consist of several parasite-derived proteins such as the Knob Associated Histidine-Rich Protein (KAHRP) that anchors the major virulence factor *P. falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1) at these protrusions (Figure 2).

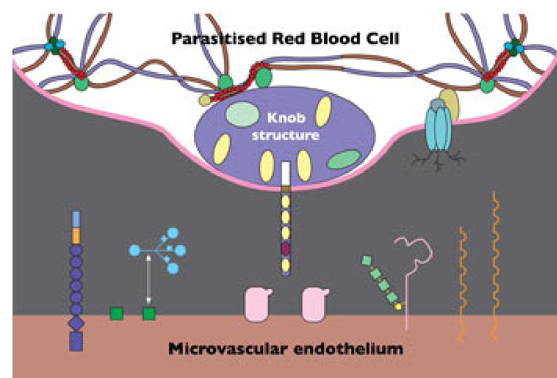

Figure 2: *Pf*EMP1 and knob structures on the surface of the infected erythrocyte. (Alan Cowman)

*Pf*EMP1 has been shown to bind to various endothelial receptors thereby mediating cytoadherence and sequestration of mature-stage infected erythrocytes in blood capillaries (Figure 3). This process contributes significantly to the clinical symptoms of malaria such as organ failure or cerebral malaria (Miller *et al.*, 2002).

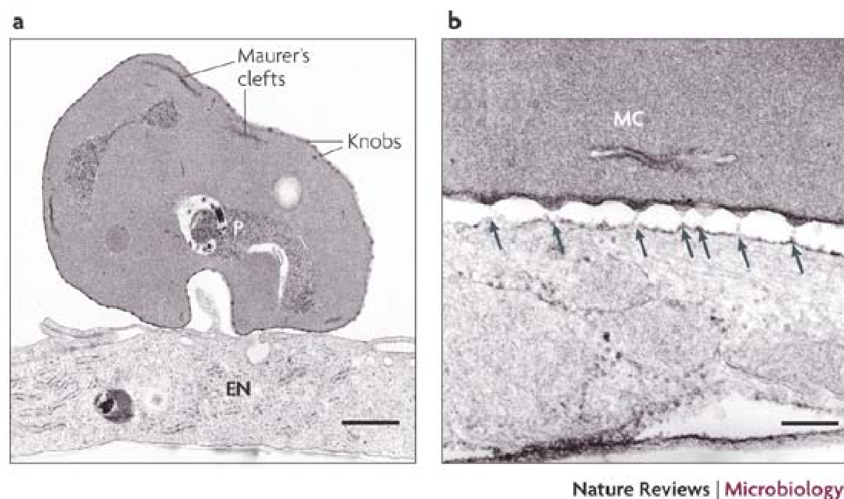

Figure 3. (a) Transmission electron micrograph of a knobby parasite (P)-infected red blood cell (RBC) adhering to the surface of a microvascular endothelial cell (EN). Scale bar is 1 µm. (b) Detail of the interface between an infected RBC and an endothelial cell showing strands of electron-dense connecting material located at knobs (arrows). Note the presence of a Maurer's cleft (MC). Scale bar is 100 nm. Images modified with permission from (Horrocks *et al.* 2005, Journal of Cell Science 118, 2507-2518) © (2005) The Company of Biologists Ltd. (Maier *et al.* 2009, Nature Reviews Microbiology 7, 341-354)

As the parasite matures the erythrocyte membrane permeability for solutes increases through the formation of **new permeability pathways** (NPPs). The origin of the proteins mediating transport across the erythrocyte membrane is still debated (Ginsburg & Stein, 2004, Staines *et al.*, 2007, Ginsburg, 2002, Huber *et al.*, 2002a, Huber *et al.*, 2002b). It is implicated that upon infection endogenous, usually silent erythrocyte transporters are activated. Recent studies substantiate the theory that NPP formation is actively mediated by proteins secreted by the parasite (Baumeister *et al.*, 2006).

Maurer's clefts are disc-like membranous structures with an electron-dense coat and an electron-lucent lumen (Atkinson *et al.*, 1988, Elford *et al.*, 1997, Kriek *et al.*, 2003, Langreth *et al.*, 1978). They appear close to the PVM and are distributed at the periphery of the erythrocyte as the parasite matures. It is debated whether Maurer's clefts are independent entities or interconnected with the TVN (Hanssen *et al.*, 2008, Tilley & Hanssen, 2008, Knuepfer *et al.*, 2005b, Spycher *et al.*, 2006,

Wickham *et al.*, 2001, Wickert *et al.*, 2004, Wickert & Krohne, 2007). However, the TVN possesses distinct markers not found in the Maurer's clefts (Haldar *et al.*, 2005, Hanssen *et al.*, 2008, Tamez *et al.*, 2008). It has been observed that the clefts are anchored to the erythrocyte cytoskeleton via electron-dense tubular structures named tethers (Waterkeyn *et al.*, 2000, Hanssen *et al.*, 2010, Hanssen *et al.*, 2008, Tilley & Hanssen, 2008, Tilley *et al.*, 2008). A hypothesis claimed that Maurer's clefts function as a surrogate, extra cellular Golgi. They are indisputably involved in the trafficking of proteins destined for the surface of the erythrocyte as shown for the transmembrane proteins *Pf*EMP1 and Subtelomeric Variable Open Reading Frame (STEVOR), as well as the soluble proteins KAHRP and *Pf*EMP3. These proteins transiently associate with the clefts before they are finally delivered to the erythrocyte membrane (Knuepfer *et al.*, 2005b, Knuepfer *et al.*, 2005a, Lavazec *et al.*, 2006, Przyborski *et al.*, 2005, Wickham *et al.*, 2001). The deletion of several Maurer's clefts resident proteins such as Skeleton Binding Protein 1 (SBP1) (Cooke *et al.*, 2006, Maier *et al.*, 2007a) or Membrane Associated Histidine-Rich Protein 1 (MAHRP1) (Spycher *et al.*, 2008) results in the disappearance of *Pf*EMP1 from the surface of the erythrocyte indicating again a major role of Maurer's clefts in protein export. EM studies suggest morphological similarities between Maurer's clefts and the Golgi of higher eukaryotes. Components of the COPII mediated vesicle-based trafficking machinery (*Pf*Sec31, *Pf*Sar1p and *Pf*Sec23) also were initially thought to be exported to the clefts (Adisa *et al.*, 2001, Albano *et al.*, 1999, Wickert *et al.*, 2003), but newer studies using transfection technology question this theory because GFP fused to *Pf*Sar1p is not exported (Adisa *et al.*, 2007). Maurer's clefts seem to represent a parasite-induced intermediate 'sorting' compartment for proteins *en route* to the erythrocyte membrane (Lanzer *et al.*, 2006, Tilley *et al.*, 2008, Wickert & Krohne, 2007). The role of Maurer's clefts in protein transport to the erythrocyte cytoplasm remains elusive.

An elucidation of the processes involved in this refurbishment initiated by early ring-stage parasites could contribute to a better understanding of the parasite's survival mechanisms and could open up a whole range of new targets for

innovative drug interventions. Spielmann and Beck have used suppression subtractive hybridization to clone genes exclusively transcribed during the *P. falciparum* ring stage to find key players of these early refurbishment events (Spielmann & Beck, 2000). In consistence with the uniqueness of the molecular events of this early parasite stage, only few genes were found with homologies to genes from other organisms. SBP1 was identified which was later shown to be a Maurer's cleft resident and essential for *Pf*EMP1 trafficking (Cooke *et al.*, 2006, Maier *et al.*, 2007a). Three members of a new gene family, the Early Transcribed Membrane Proteins (ETRAMPs), were identified and located to the PVM (Spielmann *et al.*, 2003, Spielmann *et al.*, 2006a). Among other ring stage specific genes, two histidine-rich proteins were found and called Membrane Associated Histidine-Rich Protein (MAHRP) 1 (Spycher *et al.*, 2003, Spycher *et al.*, 2006, Spycher *et al.*, 2008) and MAHRP2 (Pachlatko *et al.*, 2010).

The two histidine-rich proteins sparked our interest as such proteins have been shown to play key roles in parasite survival strategies. For instance, parasite-derived histidine-rich proteins initiate and accelerate the crystallization process of hematin *in vitro* (Papalexis *et al.*, 2001, Sullivan *et al.*, 1996). Another example is the interaction of the histidine-rich domains of KAHRP that mediate anchoring of *Pf*EMP1 in the erythrocyte membrane (Waller *et al.*, 1999). There are studies on histidine-rich proteins in other organisms. Histidine-rich glycoproteins (HRGP) identified in the plasma of many vertebrates and also in invertebrates can bind metal ions and exert pH- and Zn^{2+}-dependent antibacterial activity (Rydengard *et al.*, 2007). The histidine-rich protein Hpn identified in *Helicobacter pylori* has an affinity for nickel ions. The gram-negative bacterium *H. pylori* colonizes the human gastric mucosa leading to chronic gastritis and ulcers. Mutants lacking *hpn* were more sensitive to nickel and bismuth than the parent strain. Hpn proteins are able to detoxify rapid fluxes in metal levels (Maier *et al.*, 2007b). Another group is the family of histatins, which are small, cationic, histidine-rich peptides present in human saliva. Reports described their potent bactericidal (MacKay *et al.*, 1984) and fungicidal properties (Pollock *et al.*, 1984). These peptides form part of the

innate immune system and play an important role in maintaining oral health by limiting infections in the oral cavity.

Our aim was to shed more light onto the early processes after invasion by means of characterization of the two proteins MAHRP1 and MAHRP2. We show for MAHRP1 possible functions and requirements for trafficking to the correct destination at the Maurer's clefts (Spycher *et al.*, 2003, Spycher *et al.*, 2006) (chapter 2). In chapter 3 we address the location of MAHRP2 (see Chapter 3) and further assess interaction partners and requirements for trafficking of MAHRP2 in chapter 4.

Protein secretion

General protein secretion

As eukaryotes evolved they developed a cellular machinery for internalization and digestion of extra cellular material, targeted intracellular transport, surface remodelling, and secretion. These functions were made possible by the presence of a complex membrane-trafficking machinery. As our research interests focus on proteins exported to the cytosol of the *P. falciparum* infected erythrocyte I will briefly concentrate on the general protein secretion pathway in eukaryotes.

Most secretory proteins contain an N-terminal or internal signal peptide that directs them mostly co-translationally to the ER. Post-translational import into the ER has also been described for several secreted proteins (Klappa *et al.*, 1991, Muller & Zimmermann, 1988). Newly synthesized proteins then exit the ER at specialized exit sites from which cargo containing coat protein complex II (COPII)-coated vesicles form. These reach the Golgi apparatus where the proteins are processed and sorted towards their final destination. The transport of secreted proteins also depends on COPI-coated vesicles formed in the Golgi mediating retrograde transport to the ER and between the Golgi cisternae. Further steps in the secretion pathway involve a series of fusion events between vesicles and the plasma membrane or organelles. The specificity of these fusion processes are mediated by protein factors like SNAREs (Soluble N-ethylmaleimide-sensitive

factor Attachment protein REceptors), tethering complexes, syntaxin-binding proteins, and Rab GTPases (Jahn *et al.*, 2003). Upon fusion of a secretory vesicle with the plasma membrane soluble contents is released by exocytosis and transmembrane proteins are inserted into the plasma membrane.

Unconventional protein secretion bypasses the ER and the Golgi as described for fibroblast growth factor-2 (FGF2). FGF2 secretion might be mediated via post-translational import into secretory lysosomes or by a transporter in the plasma membrane (Backhaus *et al.*, 2004, Schafer *et al.*, 2004). Anyhow, its transport is insensitive to brefeldin A treatment which blocks classical secretion.

Protein secretion in *P. falciparum*

In *P. falciparum* infected erythrocytes we face a conceptual problem regarding protein secretion. Homologues for most of the genes of the classical secretion pathway are actually found in *P. falciparum*. Some features, however, are special and specific to this parasite. The Golgi apparatus of the parasite is highly reduced. *Apicomplexan* parasites have specialized secretory organelles called rhoptries, micronemes, and dense granules. Again, the host cell does not provide any factors for protein translocation and trafficking. Parasite proteins exported to the cytosol of the erythrocyte furthermore encounter a highly complex transport route passing through the parasite membrane, the parasitophorous vacuolar membrane, and some proteins even traverse the erythrocyte plasma membrane. Exported transmembrane proteins are either destined for the PVM, Maurer's clefts or the erythrocyte plasma membrane, whereas soluble proteins can be deposited in the erythrocyte cytosol, the lumen of Maurer's clefts or the extra-erythrocytic space. This is a unique situation and no molecular concepts exist which can be used as a conceptual basis. Additionally, a number of these secreted proteins do not even contain a recognizable N-terminal ER targeting signal peptide (Crabb *et al.*, 2010, Haase & de Koning-Ward, 2010, Lingelbach & Przyborski, 2006, Spielmann & Gilberger, 2010). Deciphering the export mechanism of these proteins is of considerable interest as such a system could be an attractive target for drug intervention (Figure 4 summarizes putative export mechanisms).

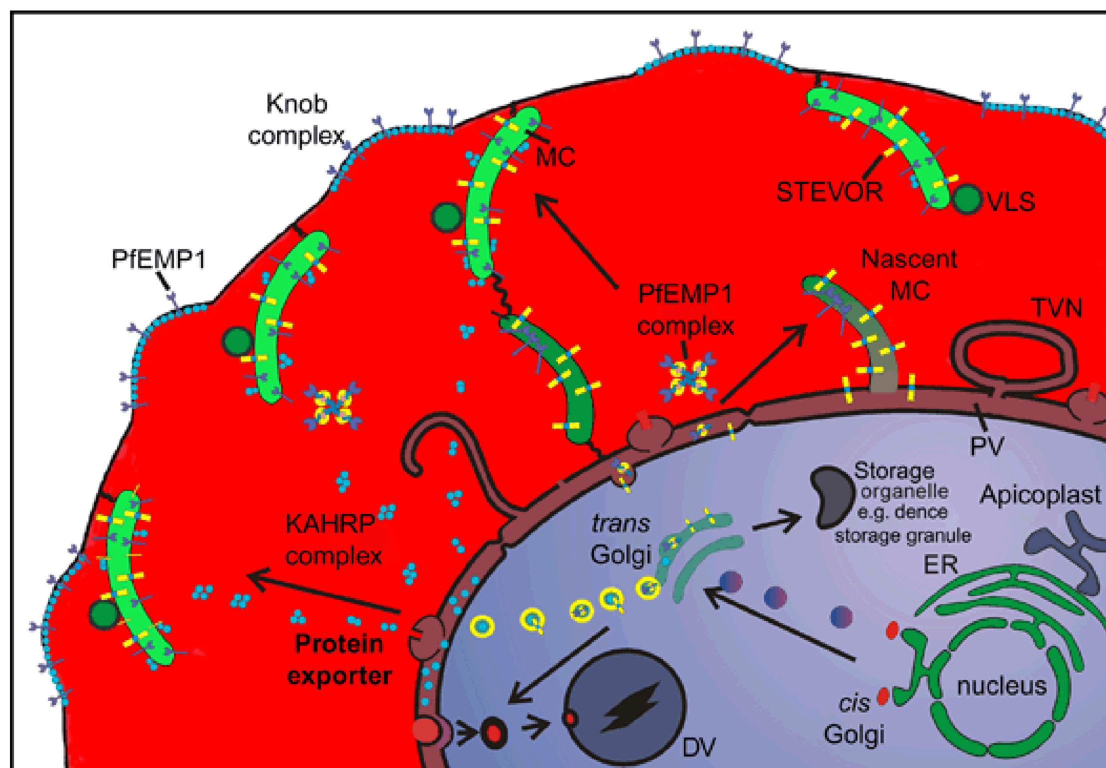

Figure 4: Diagrammatic representation of some putative trafficking pathways in the trophozoite stage of *P. falciparum*-infected erythrocytes. Soluble proteins destined for export are directed into the ER and pass through *cis*-Golgi and *trans*-Golgi compartments en route to the parasitophorous vacuole (PV). Some proteins are retrieved from the plasma membrane or diverted from the ER or Golgi to intracellular organelles, such as the digestive vacuole (DV) and the apicoplast or (in the schizont stage) to regulated secretory compartments, such as the dense granules, rhoptries and micronemes. Following transport across the PV membrane, soluble proteins (such as KAHRP) may form complexes as they diffuse across the RBC cytoplasm and interact with the cytoplasmic surface of the Maurer's clefts (MC) before redistribution to the erythrocyte membrane skeleton. *Pf*EMP1 may also be trafficked in protein complexes and may become membrane-embedded by inserting into MC from the erythrocyte cytoplasm. Integral membrane proteins destined for the MC (such as STEVOR) may be transferred to the PV membrane and then accumulate in nascent MC. Vesicle-like structures are observed in the infected erythrocyte cytoplasm; however, their role in protein trafficking is unclear. TVN, tubulovesicular network. (Tilley *et al.* Traffic Vol. 9, 2 Pages: 187-197)

A huge step in resolving the export procedure was made by the discovery that a large number of the exported proteins possess a conserved amino acid motif close to the N-terminus called the *Plasmodium* export element (**PEXEL**) (Marti *et al.*, 2004) or vacuolar transport signal (**VTS**) (Hiller *et al.*, 2004). It is a pentameric motif with the consensus sequence R/KxLxE/Q/D where x is any non-charged amino acid. Around 20 amino acids upstream of the PEXEL motif is a hydrophobic domain that mediates co- or post-translational insertion into the ER for soluble and transmembrane proteins. The description of this motif enabled the prediction of an exportome of *P. falciparum in silico* based on its presence. Around 5 to 8 % of the genome is predicted to be exported (Hiller *et al.*, 2004, Marti *et al.*, 2004, Sargeant *et al.*, 2006, van Ooij *et al.*, 2008). A large-scale gene knockout strategy combined with functional screens of genes encoding PEXEL-carrying proteins was made to prove that many of these proteins are involved in host cell refurbishment (Maier *et al.*, 2008). It was suggested that the PEXEL motif is processed in the ER by the 'pexelase' plasmepsin V (Boddey *et al.*, 2010, Russo *et al.*, 2010) whereby a new N-terminus, xE/Q/D, is generated, which in turn becomes acetylated (Boddey *et al.*, 2009, Chang *et al.*, 2008). Processed PEXEL proteins are secreted into the PV via vesicular traffic. Different speculations exist on how these proteins can be distinguished from non-exported proteins once in the PV (Boddey *et al.*, 2009, Crabb *et al.*, 2010, Haase & de Koning-Ward, 2010). It is proposed that PEXEL cleavage might facilitate an association with specific chaperones allowing specific recognition by a PVM translocation machinery. Otherwise, processed proteins could be sorted into distinct vesicles that are targeted to discrete regions of the parasite plasma membrane where the translocation machinery is present on the opposing PVM forming a bridge between the two membranes (Figure 5). Up to the present these models are theoretical only. The translocation across the PVM needs ATP (Ansorge *et al.*, 1996) and the proteins being in an unfolded state (Gehde *et al.*, 2009). Recently, the presence of a *P. falciparum* translocon for exported proteins (PTEX) has been observed at discrete foci in the PVM (de Koning-Ward *et al.*, 2009). The complex is of parasitic origin and is found on the inside of the PVM. The complex comprises an ATPase (HSP101), the predicted pore forming exported protein 2 (EXP2) and the potential regulators TRX2, PTEX150,

and PTEX88. The complex has been shown to interact with PEXEL proteins; however, its evolutionary origin is not apparent. This could be explained by the fact that PEXEL-containing proteins are unique to malaria parasites. (Figure 5 summarizes PEXEL mediated export.)

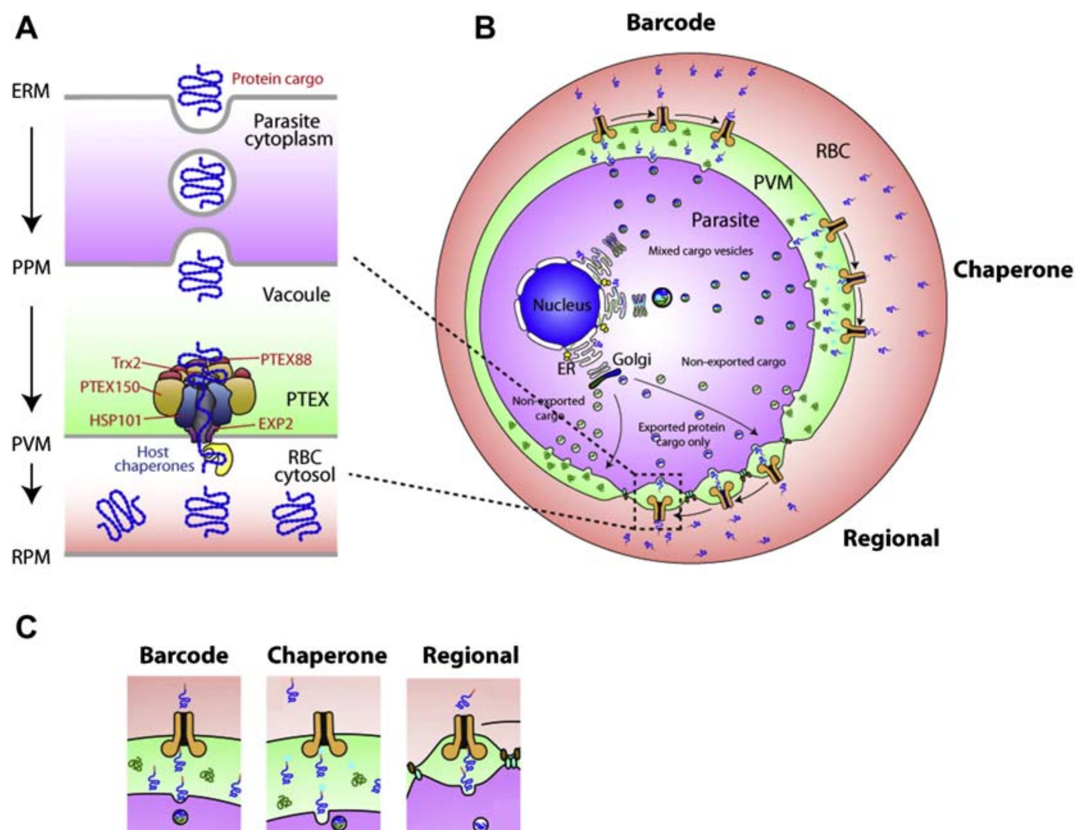

Figure 5: Diagram of proposed *Plasmodium* export element (PEXEL) protein export models in *Plasmodium*-infected red blood cells (RBCs). (A) Parasite PEXEL proteins are processed for export in the endoplasmic reticulum (ER) and then migrate to plasma membrane by vesicular transport. Once deposited into the vacuole space the PEXEL proteins are probably unfolded and extruded through a pore across the parasitophorous vacuole membrane (PVM) into the erythrocyte cytosol by the *Plasmodium* translocon of exported proteins (PTEX) complex. To date five proteins have been identified in the PTEX complex and their hypothetical arrangement is shown. On the RBC side of the PVM export is probably assisted by erythrocyte chaperones. (B and C) Three models, the so-called barcode, chaperone and regional models have been proposed to explain middle stages of protein export from the ER to the PTEX translocon. In the barcode and chaperone models PEXEL proteins (blue) exit the ER/Golgi in bulk

flow vesicles mixed with secreted non-exported proteins (green) and are deposited into the vacuole. In the barcode model just a few N-terminal amino acids and a flexible spacer are required for recognition by PTEX for translocation into the erythrocyte cytosol. In the chaperone model PTEX recognition is via a chaperone (cyan) attached to the PEXEL protein. In the final regional model PEXEL proteins are sorted from bulk flow non-exported proteins in the ER/Golgi and are packaged into specialised vesicles that dock and empty into specialised export compartments that contain PTEX (boxed) which exports them into the erythrocyte cytosol. ERM, endoplasmic reticulum membrane; PPM, parasite plasma membrane and RPM, red blood cell plasma membrane. (Crabb *et al.* 2010, International Journal for Parasitology, Vol. 40, 5 Pages 509-513)

Even though the identification of the PEXEL motif has expanded the understanding of protein export in *P. falciparum* to a great extent there are exceptions to the rule. Some well characterized proteins exported to Maurer's clefts are PEXEL-negative and lack a common signal sequence. These proteins are called PEXEL-negative exported proteins (**PNEPs**). Current predictions on the 'exportome' are therefore incomplete. The PNEP proteome includes Maurer's cleft resident proteins MAHRP1, SBP1, REX1 and REX2 (Blisnick *et al.*, 2000, Haase *et al.*, 2009, Hawthorne *et al.*, 2004, Spielmann *et al.*, 2006b, Spycher *et al.*, 2003). MAHRP1, SBP1 and REX2 share some structural similarities in that they all contain a single transmembrane domain. REX1 also contains a hydrophobic stretch which is, however, more of a recessed signal peptide than a transmembrane domain. It is suggested that all these proteins enter the classical secretory pathway because ER intermediates have been found for all of them (Dixon *et al.*, 2008, Haase *et al.*, 2009, Saridaki *et al.*, 2008, Spycher *et al.*, 2006). Lately, several research groups tried to identify sequence requirements for the export of PNEPs, yet, no common motif or perceptible sequence was found promoting their export. The transmembrane domain seems to be essential, most likely for entering the ER and secretory pathway (Haase *et al.*, 2009, Saridaki *et al.*, 2009, Spycher *et al.*, 2006). For MAHRP1 and SBP1 the transmembrane domain and the second half of the N-terminal domain are sufficient for export and transfer to the Maurer's clefts (Saridaki *et al.*, 2009, Spycher *et al.*, 2006). REX1 is translocated across the PV

membrane as a soluble protein, which then docks at the Maurer's clefts (Dixon *et al.*, 2008). The N-terminal hydrophobic stretch plus an additional 10 amino acids is sufficient to direct export of this protein, while a coiled-coil region in the C-terminal domain is needed for binding to the Maurer's clefts (Dixon *et al.*, 2008). In contrast, the first 10 amino acids of the N-terminal domain and the transmembrane domain are sufficient to target REX2 to Maurer's clefts (Haase *et al.*, 2009). Interestingly, the first 10 amino acids of REX2 can be substituted with the first 10 amino acids of SBP1 or MAHRP1, suggesting a similar means of transport although they are not required for SBP1 or MAHRP1 export.

It is still absolutely unclear whether all exported proteins travel the same route to the erythrocyte cytosol or if multiple trafficking pathways are taken. The number of predicted PEXEL proteins in other *Plasmodium* species is much smaller compared to *P. falciparum* (Sargeant *et al.*, 2006). PEXEL proteins were expected to add significantly to host cell modifications in *P. falciparum* and extensive modifications are also observed in other species (Bray & Garnham, 1982, Mackenstedt *et al.*, 1989). This may mean that the role of PNEPs in the refurbishment of the host cell by these parasites is more eminent. This becomes more evident as half of the *P. vivax* VIR surface antigen family and the two largest variant surface antigen families in *P. knowlesi*, SICA and KIR, lack a typical PEXEL motif (Carlton *et al.*, 2008, Pain *et al.*, 2008) and thus give evidence for other transport mechanisms in *Plasmodia species*.

Aiming at further delineation of the export mechanism of PEXEL-negative exported proteins we have focused on the sequence requirements of the recently described PNEP MAHRP2 (see Chapters 3 and 4).

Outline of this thesis

The aim of this thesis was to shed more light on the processes induced by *P. falciparum* early after invasion that are involved in host cell refurbishment. Two proteins, the Membrane Associated Histidine-Rich Proteins 1 and 2 (MAHRP1 and MAHRP2) were previously found to be expressed at this early ring stage (Spielmann & Beck, 2000). Here, both proteins were further characterized. MAHRP1 is a Maurer's clefts resident protein (Spycher *et al.*, 2003). The *mahrp1* gene was genetically disrupted to study possible functions of the protein in the genetic knock down parasite (chapter 2). The location of MAHRP2 was addressed by immunofluorescence and electron microscopy (chapter 3). Solubility characteristics of MAHRP1 and MAHRP2 are also shown in chapter 3. Possible protein interaction partners identified in pull down experiments with recombinant MAHRRP2 are described in chapter 4. We failed to genetically ablate the *mahrp2* gene indicating an essential function for MAHRP2 in parasite survival strategies. In chapter 4 it is shown that MAHRP2 protein levels could be dramatically reduced through tagging with the FKBP destabilizing domain. Both MAHRPs are PEXEL-negative exported proteins and sequence requirements for trafficking of MAHRP1 (Spycher *et al.*, 2006) and MAHRP2 were found by transfection of parasites with plasmids expressing truncated versions of the protein fused to a GFP-tag. These results are presented in chapter 3 and 4.

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Chapter 2

The Maurer's cleft protein MAHRP1 is essential for trafficking of *Pf*EMP1 to the surface of *Plasmodium falciparum*-infected erythrocytes

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Summary

During the intra-erythrocytic development of *Plasmodium falciparum*, the parasite modifies the host cell surface by exporting proteins that interact with or insert into the erythrocyte membrane. These proteins include the principal mediator of cytoadherence, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). To implement these changes, the parasite establishes a protein-trafficking system beyond its confines. Membrane-bound structures called Maurer's clefts are intermediate trafficking compartments for proteins destined for the host cell membrane. We disrupted the gene for the membrane-associated histidine-rich protein 1 (MAHRP1). MAHRP1 is not essential for parasite viability or Maurer's cleft formation; however, in its absence, these organelles become disorganized in permeabilized cells. Maurer's cleft-resident proteins and transit cargo are exported normally in the absence of MAHRP1; however, the virulence determinant, PfEMP1, accumulates within the parasite, is depleted from the Maurer's clefts and is not presented at the red blood cell surface. Complementation of the mutant parasites with *mahrp1* led to the reappearance of PfEMP1 on the infected red blood cell surface, and binding studies show that PfEMP1-mediated binding to CD36 is restored. These data suggest an important role of MAHRP1 in the translocation of PfEMP1 from the parasite to the host cell membrane.

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Introduction

The apicomplexan parasite *Plasmodium falciparum* causes the most severe form of human malaria. The blood stages are responsible for most of the clinical symptoms of the disease and the associated mortality. While most intracellular pathogens interact with an active host cell, the malaria parasite develops within a cell that is devoid of all organelles and any protein-trafficking machinery. The parasite resides in a parasitophorous vacuole (PV) encircled by a limiting membrane and modifies its host cell by establishing membranous structures in the red blood cell (RBC) cytoplasm. These comprise a tubovesicular network extending from the PV membrane (PVM) (Behari and Haldar, 1994; Elmendorf and Haldar, 1994) and disc-shaped structures called Maurer's clefts at the RBC periphery (Atkinson *et al.*, 1988; Elford *et al.*, 1997; Lanzer *et al.*, 2006). In addition, the parasite modifies the surface of the infected RBC with exported proteins that contribute to the virulence of *P. falciparum* (reviewed in Kyes *et al.*, 2001). A key protein in this process is the variant surface antigen, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates adherence of infected RBCs to the endothelial cells of the host via host receptors such as CD36 (Pasloske *et al.*, 1993; Baruch *et al.*, 1995; 1996; Voss *et al.*, 2006). PfEMP1 is presented at knob structures on the RBC surface. Its C-terminal domain is oriented towards the host cell cytoplasm and interacts with the knob-associated histidine-rich protein (KAHRP) (Pologe *et al.*, 1987).

As mature RBCs lack cellular trafficking machinery, the parasite establishes its own secretory system for exporting proteins through the PVM and across the host cell cytoplasm. The mechanisms and molecular apparatus

involved in this process are not completely understood. A classical or recessed signal sequence is sufficient to direct proteins into the parasite's endoplasmic reticulum (ER) with default release into the PV (Waller *et al.*, 1998; Wickham *et al.*, 2001; Adisa *et al.*, 2003). Onward transport across the PVM requires an additional signature that was recently identified and termed the *Plasmodium* export element (PEXEL; Marti *et al.*, 2004) or vacuolar transport signal (VTS) (Hiller *et al.*, 2004).

A number of exported soluble proteins, such as KAHRP and the *P. falciparum* erythrocyte membrane protein 3 (PfEMP3), transiently associate with the cytoplasmic surface of the Maurer's clefts before redistribution to their final destinations at the RBC membrane (Wickham *et al.*, 2001; Knuepfer *et al.*, 2005a). Indeed, a recent report suggests that Maurer's clefts are secretory organelles that concentrate virulence proteins for delivery to the host erythrocyte membrane (Bhattacharjee *et al.*, 2008). The integral membrane protein, PfEMP1, also accumulates at Maurer's clefts before transfer to the RBC membrane (Kriek *et al.*, 2003; Knuepfer *et al.*, 2005b). These data suggest that the Maurer's clefts perform a role as an intermediate compartment for proteins *en route* to the RBC membrane.

The Maurer's clefts have a number of resident integral membrane proteins (Vincensini *et al.*, 2005). These include the membrane-associated histidine-rich protein 1 (MAHRP1; Spycher *et al.*, 2006), skeleton binding protein 1 (SBP1; Blisnick *et al.*, 2000), the Maurer's cleft Pfmc2-TM proteins (Sam-Yellowe *et al.*, 2004), the subtelomeric variable open reading frame (STEVORs) (Kaviratne *et al.*, 2002; Przyborski *et al.*, 2005) and the ring exported proteins (REX1 and REX2; Hawthorne *et al.*, 2004; Spielmann *et al.*, 2006). Knockout of SBP1 prevents export of PfEMP1 to the surface of infected RBCs, revealing the potential importance of the Maurer's cleft-resident proteins (Cooke *et al.*, 2006; Maier *et al.*, 2006).

We have previously described the Maurer's cleft-resident protein, MAHRP1, which is exclusively transcribed in ring stages (Spycher *et al.*, 2003; 2006; Spielmann *et al.*, 2006). It is a transmembrane protein with a histidine-rich C-terminal domain, oriented towards the RBC cytoplasm (Spycher *et al.*, 2003). MAHRP1 contains neither a classical signal sequence nor an obvious PEXEL motif. Analysis of a series of GFP fusions of MAHRP1 fragments showed that the transmembrane domain directs the protein into the ER, while additional as-yet-unidentified motifs within the N-terminal region are required for translocation of the protein to the Maurer's clefts (Spycher *et al.*, 2006).

To understand the function of MAHRP1, we have generated deletion mutants of the *MAHRP1* gene in *P. falciparum* (3D7 strain). Maurer's clefts are still formed in the absence of MAHRP1, but display differences in morphology and fragility in permeabilized cells. Interestingly, export of PfEMP1 is compromised and this protein accumulates within the confines of the plasma membrane/PVM interface and no PfEMP1 is detected on the surface of the infected RBC. By contrast, other exported proteins, such as KAHRP and PfEMP3, are still correctly trafficked. We complemented the deletion mutants with *mahrp1* and showed by immunofluorescence and trypsin digestion assays that PfEMP1 is again trafficked to the host cell membrane. We performed CD36 binding assays that confirm that PfEMP1 is functionally present at the RBC membrane of the complemented strain. These data suggest that MAHRP1 plays an essential role in the export of PfEMP1 from the parasite to the host cell surface.

Results

Disruption of the MAHRP1 locus

MAHRP1 (plasmoDB *MAL13P1.413*) is a transmembrane protein that is expressed during ring stages but present throughout the erythrocyte life cycle, and exported beyond the parasite's confines into parasite-derived structures known as Maurer's clefts (Spycher *et al.*, 2006). To determine the function of MAHRP1 and to better understand the role of Maurer's clefts in protein export, we disrupted the expression of endogenous *MAHRP1* in *P. falciparum* (3D7 strain). We generated the plasmid *pTK_M1* containing the human dihydrofolate reductase coding sequence (*hdhfr*) cassette flanked by *MAHRP1* 5′ and 3′ flanks. Integration of the drug-resistance cassette into the *MAHRP1* locus is expected to disrupt the coding region (see schematic representation, Fig. 1).

Southern blot analysis of genomic DNA of both wild type and transfected parasites with a labelled 3′ flank PCR product of *MAHRP1* revealed single cross-over recombination into the *MAHRP1* locus at the 5′ UTR, as shown in Fig. 1A. Western blot analysis using a polyclonal mouse serum against the C-terminal region of MAHRP1 revealed a band at approximately 30 kDa (corresponding to the correct size of MAHRP1) in the parent line (Fig. 1B, left-hand lane). MAHRP1 could not be detected in Pf3D7ΔM1 transfectants (Fig. 1B, right-hand lane), showing that disruption of the *MAHRP1* locus ablates MAHRP1 expression.

Maurer's clefts are formed in the absence of MAHRP1

To confirm the absence of MAHRP1 in Pf3D7ΔM1 at the cellular level, we performed immunofluorescence microscopy on non-synchronized acetone–methanol-fixed infected RBCs as shown in Fig. 2A. When the parent line was probed with mouse anti-MAHRP1 serum, a punctate pattern was observed in the RBC cytoplasm consistent with Maurer's cleft labelling (Fig. 2A, upper panel). No

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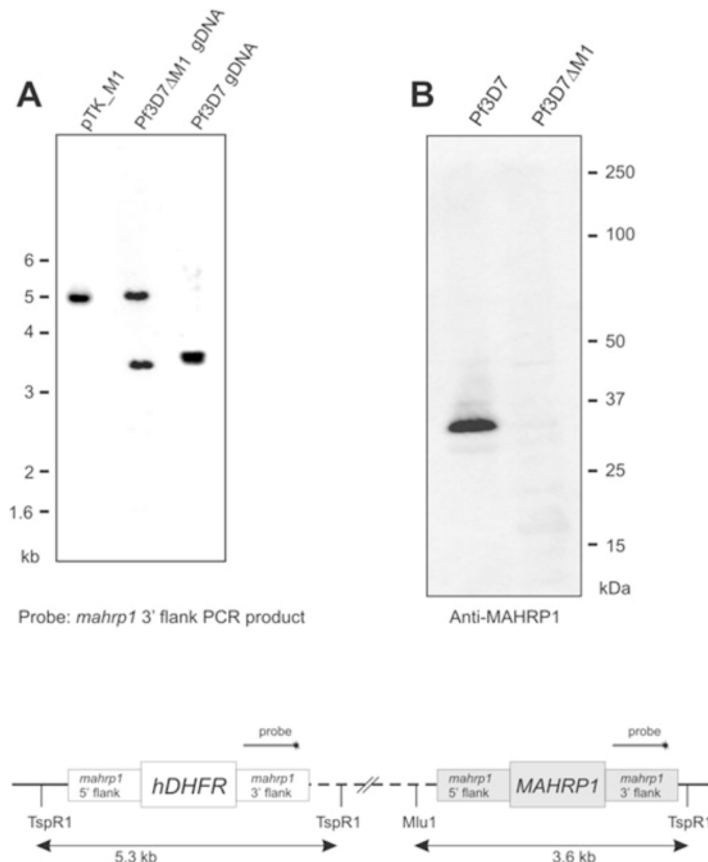

Fig. 1. *MAHRP1* disruption analyses.
A. Southern blot analysis confirms integration of *pTK_M1* into the *MAHRP1* locus. Genomic DNA and the transfection plasmid *pTK_M1* were digested with *TspR*1 and *Mlu*1 restriction enzymes and probed with a labelled 3′ flank PCR product. The predicted sizes are 5137 (plasmid *pTK_M1*), 3792 (wild-type 3D7), 5333 and 3590 bp (5′ single cross-over integrant Pf3D7ΔM1). The schematic diagram depicts the disruption of the endogenous promoter by a 5′ single cross-over event.
B. Western blot confirms ablation of MAHRP1 expression in Pf3D7ΔM1-infected RBCs. Saponin-insoluble material from the parental 3D7 and Pf3D7ΔM1 parasites was subjected to SDS-PAGE, transferred to nitrocellulose and probed with mouse anti-MAHRP1 antiserum.

labelling of Maurer's clefts was observed in Pf3D7ΔM1, also confirming the disruption of the *MAHRP1* locus (Fig. 2A, lower panel).

To determine whether the formation or composition of the Maurer's clefts was altered in the absence of MAHRP1, we looked at three different Maurer's cleft-resident proteins. Antibodies against SBP1 (Blisnick *et al.*, 2000) (Fig. 2B) labelled punctate structures in both the parent line and the Pf3D7ΔM transfectants, showing that both strains still express and export SBP1 to the Maurer's clefts. Similarly, anti-REX1 (Hawthorne *et al.*, 2004) and antiserum against another Maurer's cleft-resident protein, termed membrane-associated histidine-rich protein-2 (MAHRP2, Pf13_0276) (E. Pachlatko, A. Müller, and H.-P. Beck, unpubl. data), labelled Maurer's clefts in both parasite lines (Fig. 2C and D). These data show that the absence of MAHRP1 does not impair export of other Maurer's cleft-resident proteins and that MAHRP1 is apparently not essential for the formation of Maurer's clefts.

PfEMP1 accumulates within the confines of the PVM in Pf3D7ΔM1 mutants

PfEMP1 is trafficked via the Maurer's clefts to the RBC membrane where it is inserted, with its variable N-terminal domain exposed at the surface and able to interact with host cell ligands such as CD36. PfEMP1 is trafficked through the ER, the plasma membrane, the PVM and the Maurer's clefts; however, details of the mode of trafficking are currently debated (Knuepfer *et al.*, 2005b; Marti *et al.*, 2005; Lanzer *et al.*, 2006; Spycher *et al.*, 2006). To assess whether MAHRP1 plays a role in the export of PfEMP1, we compared the immunofluorescence pattern obtained using anti-PfEMP1 on the parent line and the transfectants with the disrupted *MAHRP1* gene (Fig. 3A). In the parent line, PfEMP1 showed a characteristic accumulation at the Maurer's clefts. In contrast, PfEMP1 accumulated within the confines of the parasite plasma membrane/PVM in Pf3D7ΔM1-infected RBCs. This pro-

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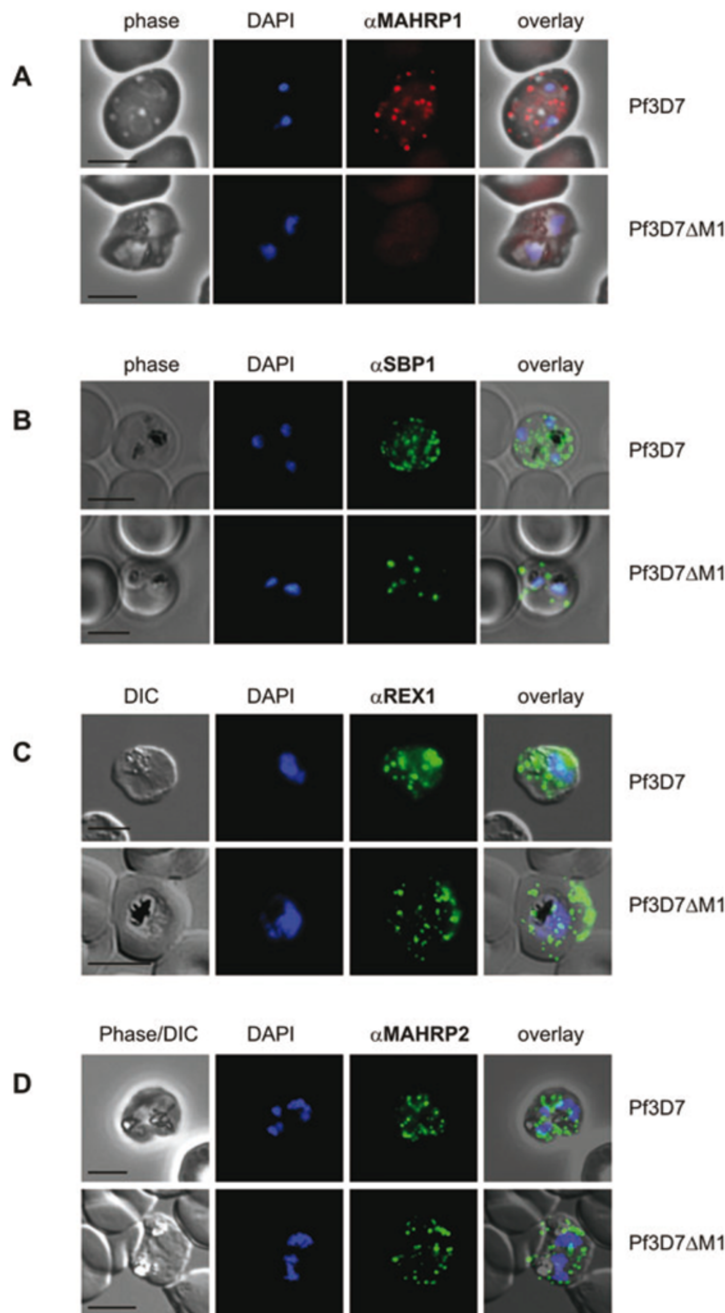

Fig. 2. Immunofluorescence microscopy of Maurer's cleft-resident proteins. Smears of infected RBCs were fixed with acetone/methanol and probed with a series of antibodies recognizing Maurer's cleft-resident proteins. Each row represents a phase (or DIC) image, a DAPI stain of the parasite nuclei, the immunofluorescence signal and an overlay of this image with the transmission image. The top row of each panel is wild-type 3D7; the second row is Pf3D7ΔM1.
A. MAHRP1.
B. SBP1.
C. REX1.
D. MAHRP2. Scale bar = 5 μm.

vides strong evidence that MAHRP1 plays an important role in trafficking of PfEMP1 beyond the PVM.

In order to determine whether the trafficking pathway of other proteins was disrupted in the absence of MAHRP1, we examined the locations of proteins that are known to transiently associate with the Maurer's clefts before associating with the RBC membrane (KAHRP and PfEMP3). KAHRP displayed a fluorescence profile consistent with a

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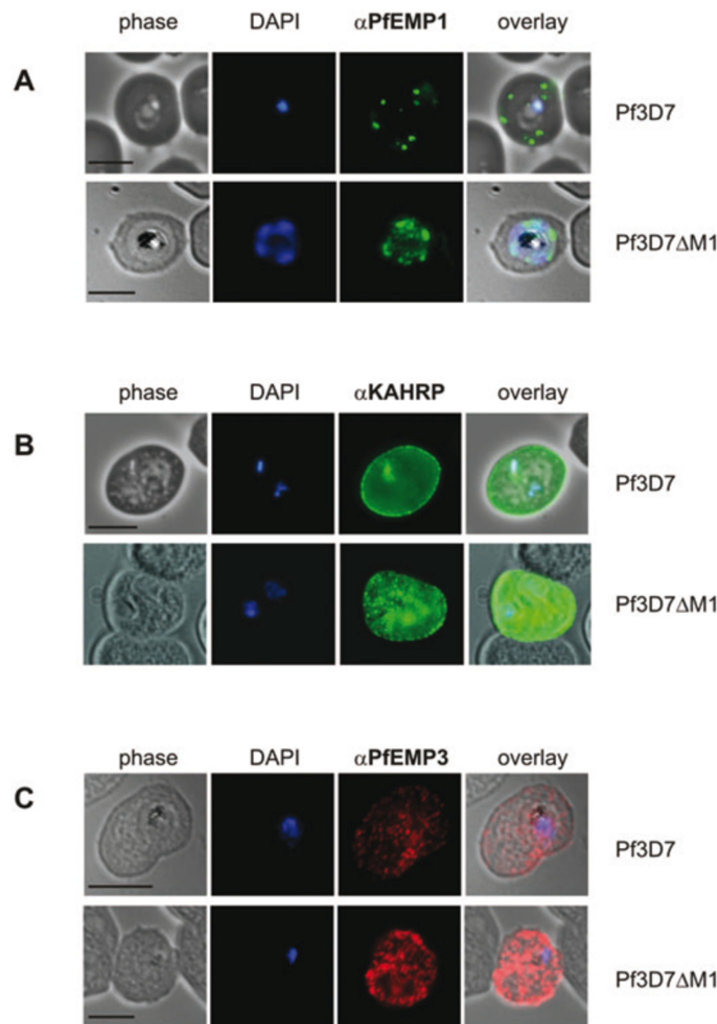

Fig. 3. Immunofluorescence microscopy of exported proteins. Smears of RBCs infected with parental wild-type 3D7 (top row) and Pf3D7ΔM1 (bottom row) were probed with antibodies recognizing proteins exported to the RBC membrane via Maurer's clefts.
A. PfEMP1.
B. KAHRP.
C. PfEMP3. Scale bar = 5 μm.

location at the RBC membrane (Pologe *et al.*, 1987) in both wild type and Pf3D7ΔM1 parasites (Fig. 3B), indicating correct trafficking to knob structures. PfEMP3 showed a heterogeneous distribution over the host cell (Fig. 3C) in both lines consistent with previous reports (Pasloske *et al.*, 1993), showing that this protein is correctly exported to the host cell cytoskeleton. Thus, both KAHRP and PfEMP3 appear to be exported independently of MAHRP1.

In wild-type parasites, a portion of the PfEMP1 population is transported to the RBC surface and inserted into the RBC membrane, allowing the extracellular N-terminal domain to be cleaved with trypsin (Kriek *et al.*, 2003; Maier *et al.*, 2006). To confirm the effect of MAHRP1 deletion on PfEMP1 trafficking, we used Western blotting of control infected RBCs and cells that had been treated with trypsin. Analysis of control 3D7-infected RBCs with an antibody recognizing the conserved C-terminal domain of PfEMP1 reveals a Triton X-100-insoluble protein with an apparent molecular weight greater than 250 kDa corresponding to full-length PfEMP1 (thick arrow). Trypsin treatment of the RBC infected parent line leads to the production of cleavage migrating as a doublet of about 80 kDa (thin arrows) which is not present in untreated cells, as described previously for this cell line (Maier *et al.*, 2007) (Fig. 4). This band is not present in uninfected RBCs (right-hand lane). In RBCs infected with the MAHRP1-disrupted line, Pf3D7ΔM1 (Fig. 4, lanes 3 and

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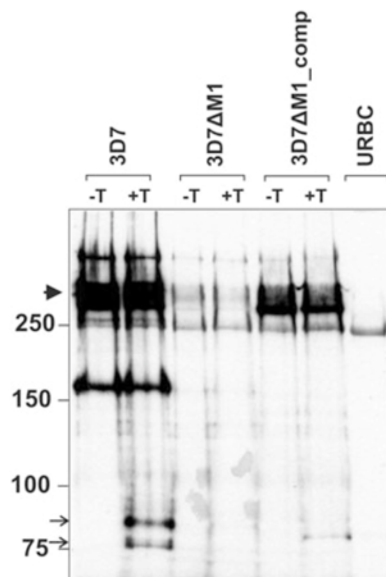

Fig. 4. Western blot analysis of PfEMP1 in 3D7, Pf3D7ΔM1 and Pf3D7ΔM1_comp-infected RBCs. Infected RBCs were trypsinized or mock-treated, extracted with Triton X-100, then subjected to SDS-PAGE and Western blotting and probed with mouse anti-PfEMP1. (–T) represents infected RBCs treated with PBS only, (+T) represents infected RBCs treated with trypsin. Full-length PfEMP1 (thick arrow) is lost during sample preparation in Pf3D7ΔM1 parasites. A cleaved product of PfEMP1 (~80 kDa, thin arrows) is observed in the parental wild-type 3D7 line and in the complemented strain, Pf3D7ΔM1_comp. URBC, uninfected RBCs.

4), there is a substantial loss of the Triton X-100-insoluble protein migrating at 250 kDa. This probably reflects the fact that PfEMP1 is Triton-soluble during trafficking within the parasite and only becomes detergent-insoluble when it reaches the Maurer's clefts or RBC membrane (Kriek *et al.*, 2003). A similar result has been observed previously in SBP1 deletion mutants (Cooke *et al.*, 2006). Consistent with this, there is no degradation product visible in the trypsin-treated Pf3D7ΔM1-infected RBCs. The data further indicate that PfEMP1 is not exported to the Maurer's clefts or the infected RBC surface as indicated by immunofluorescence, and demonstrate the important role of MAHRP1 in export and trafficking of PfEMP1 to the RBC surface.

Ultrastructure of Maurer's clefts in wild-type parasites and parasites with the MAHRP1 locus disrupted

We examined the morphology of Maurer's clefts in parent and MAHRP1-disrupted parasites. The Maurer's clefts in the parent line are characterized by a translucent lumen and an electron-dense coat (Fig. 5A). Maurer's clefts are also evident in parasites with the disrupted MAHRP1 locus (Fig. 5B and C), and there is no obvious difference in the number of Maurer's clefts in these parasites. We have previously developed a method using EqtII to release haemoglobin from infected RBCs to reveal additional details of membranous structures in the host cell cytoplasm and to enable labelled antibodies to bind to epitopes exposed to the RBC cytoplasm (Jackson *et al.*, 2007; Hanssen *et al.*, 2008). In RBCs infected with 3D7 parasites, the Maurer's clefts were observed as thin cisternal structures with an electron-dense coat (Fig. 5D and G) or as flattened structures in transverse sections (Fig. 5E). By contrast, in EqtII-permeabilized Pf3D7ΔM1-infected RBCs, the Maurer's clefts were fragmented or swollen (Fig. 5F, H and I).

We examined the labelling of the Maurer's clefts with antibodies recognizing SBP1 and PfEMP1. Anti-SBP1 antibodies (Blisnick *et al.*, 2000) decorated both the typical Maurer's clefts of 3D7 parasites and the swollen Maurer's clefts of Pf3D7ΔM1 parasites (Fig. 5G–I). An antibody recognizing the cytoplasmic domain of PfEMP1 (Kriek *et al.*, 2003) recognized PfEMP1 associated with Maurer's clefts (Fig. 5D and E) and occasionally with the RBC membrane (not shown) in the parent line. By contrast, no gold particles were observed in association with the Maurer's clefts in MAHRP1-disrupted parasites (Fig. 5F). We observed occasional gold particles associated with the PVM and the RBC membrane (Fig. 5F); however, the labelling was very weak and may be due to non-specific binding of the antibody.

To obtain information about the 3D structure of the Maurer's clefts, we have undertaken a tomographic reconstruction of electron micrographs (employing 300 nm sections) of permeabilized infected RBCs (Fig. 5J and K). In wild-type parasites, Maurer's clefts are easily distinguished from the double membranes of the parasite plasma membrane and PVM by their electron-dense coat and by the labelling with SBP1. The rendered images reveal the morphology of the flattened cisternae of the Maurer's clefts of 3D7 parasites. By contrast, the Maurer's clefts of Pf3D7ΔM1 parasites were more fragmented and had a much higher width to length ratio. In order to quantify these apparent differences in Maurer's cleft morphology, we measured the ratio of the width (x) to the length (y) of Maurer's clefts in a series of electron tomograms. We examined 17 different Maurer's clefts each for both wild-type parasites and the deletion mutant, and measured the x/y ratio at the region of maximum length. The ratio was 12.6 ± 3.1 (SD) for 3D7 and 1.6 ± 0.7 (SD) for Pf3D7ΔM1.

mahrp1 *complementation of Pf3D7ΔM1 restores MAHRP1 expression and location*

To confirm the role of MAHRP1 in the trafficking of PfEMP1 beyond the parasite's confines, we

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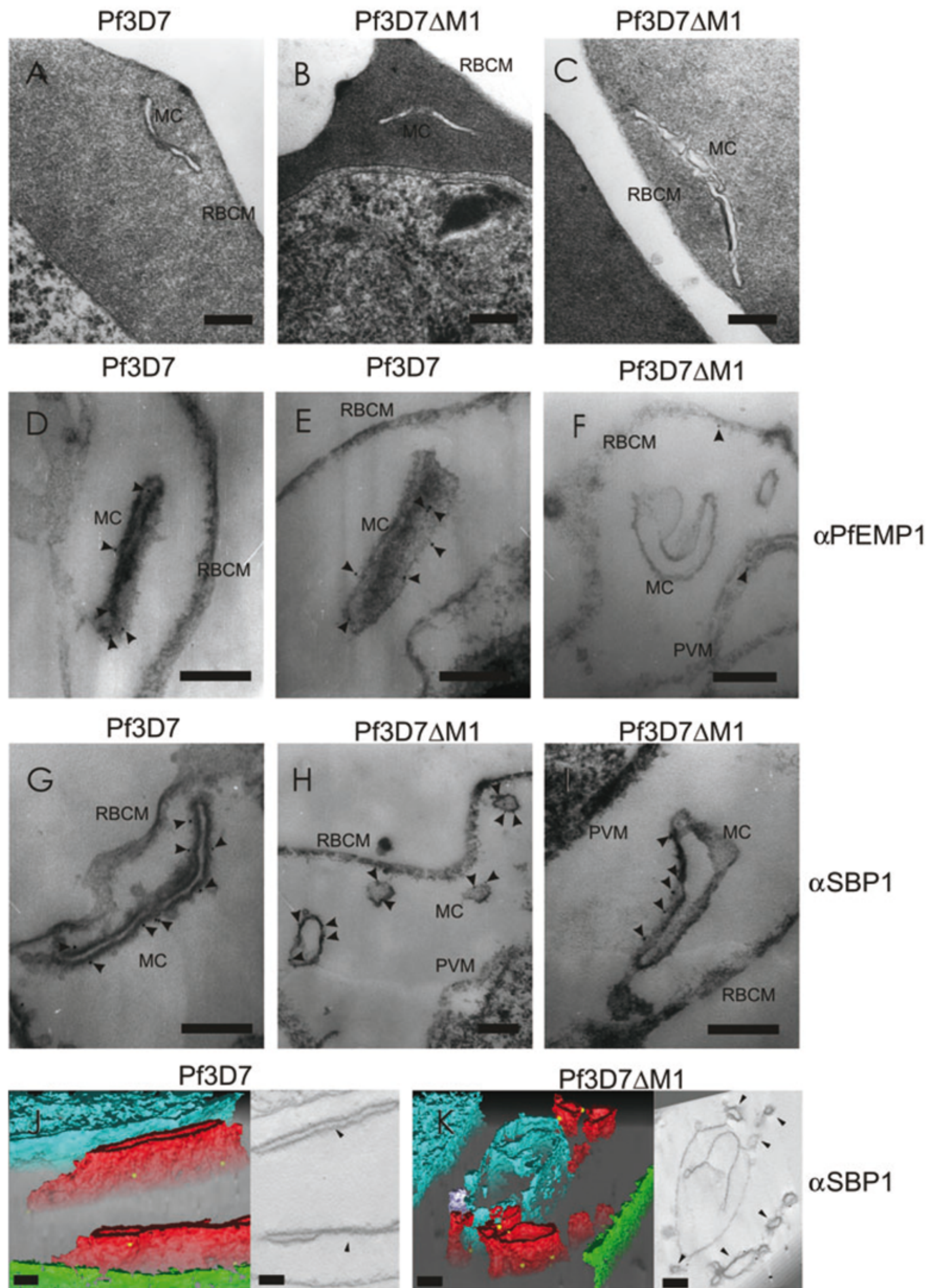

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Fig. 5. EM of 3D7 and Pf3D7ΔM1-infected RBCs.
A–C. Sections through RBCs containing mid-trophozoite stage wild-type 3D7 parasite (A) or Pf3D7ΔM1 transfectant (B and C), showing Maurer's clefts (MC) underlying the RBC membrane (RBCM).
D–F. Paraformaldehyde-fixed EqtII-permeabilized RBCs infected with parent (D and E) or Pf3D7ΔM1 (F) parasites were labelled with anti-PfEMP1. In 3D7-infected RBCs, the Maurer's clefts appear as long slender cisternae and the gold particles are associated with these structures. The MAHRP1-disrupted parasites are not labelled with anti-PfEMP1 although occasional gold particles are observed associated with the PVM and the cytoplasmic surface of the RBC membrane (F, arrowheads).
G–I. EqtII-permeabilized RBCs infected with parent (G) or Pf3D7ΔM1 (H and I) parasites were labelled with anti-SBP1 antiserum followed by protein A gold (6 nm conjugate). In 3D7-infected RBCs, the gold particles are associated with the electron-dense coat of the Maurer's cleft (G, arrowheads). In Pf3D7ΔM1-infected RBCs (H and I), the Maurer's clefts appear swollen and fragmented, but are still labelled with anti-SBP1 (arrowheads). Bars are 100 nm.
J and K. Semi-thick sections (300 nm) of EqtII-permeabilized 3D7 (J) and Pf3D7ΔM1 (K) parasites were observed with a Tecnai G2 TF30 at an accelerating voltage of 200 keV. A tilt series from −60 to 60 degrees with an increment of 2 degrees was collected. The tomograms were generated using the IMOD package. The entire tomograms were used to generate the models and drive the rendering process. (red – Maurer's cleft, green – RBC membrane, blue – PVM). The gold particles are located on the Maurer's clefts and depleted from the RBC membrane and PVM. Bars: 100 nm.

complemented Pf3D7ΔM1 with a plasmid harbouring *mahrp1*, resulting in a strain referred to as Pf3D7ΔM1_comp. To check for the presence of MAHRP1 in the complemented strain, we performed immunoblot analyses probing with anti-MAHRP1 (see Fig. 6A, left blot). A band of approximately 40 kDa was detected in Pf3D7ΔM1_comp (lane 1) migrating a few kDa higher than endogenous MAHRP1 in the wild-type strain (lane 3). This is consistent with the predicted size of the HA-tagged version of MAHRP1 in Pf3D7ΔM1_comp being slightly higher than that of endogenous MAHRP1. As a control, a protein lysate of Pf3D7ΔM1 was run on the same blot and no MAHRP1 was detected (lane 2). To confirm that MAHRP1 in Pf3D7ΔM1_comp is a product of plasmid-encoded HA-tagged MAHRP1, we loaded the same samples as in the left blot but probed with an anti-HA antibody. As shown in the right blot in Fig. 6A, a band is clearly detectable in Pf3D7ΔM1_comp (lane 4), but no bands are detected in Pf3D7ΔM1 (lane 5) or Pf3D7 (lane 6).

To confirm the presence of MAHRP1 in Pf3D7DM1_comp on the cellular level, we probed fixed cells with an antibody against MAHRP1 (Fig. 6B). The

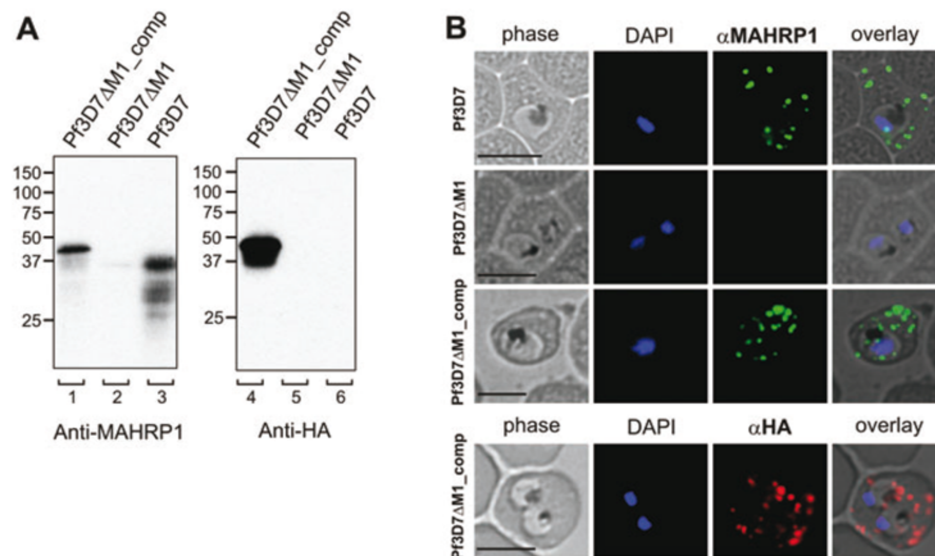

Fig. 6. MAHRP1 expression and location are restored after complementation of the MAHRP1-deficient strain, Pf3D7ΔM1.
A. Western blot analyses of the presence of MAHRP1 in Pf3D7ΔM1_comp, Pf3D7ΔM1 and wild-type Pf3D7 by probing with anti-MAHRP1 (left blot) and anti-HA-tag antibody (right blot). No MAHRP1 is detected in the MAHRP1-disrupted strain Pf3D7ΔM1, whereas there is a MAHRP1-specific band in Pf3D7 and Pf3D7ΔM1_comp (left blot). The complemented strain expresses a HA-tagged MAHRP1 which is not present in the wild type and Pf3D7ΔM1.
B. Immunofluorescence assays of fixed RBCs infected with Pf3D7ΔM1_comp, Pf3D7ΔM1 or wild-type 3D7 probed with anti-MAHRP1 (rows 1–3) and anti-HA (row 4). Each row represents a bright field image, a DAPI stain of the parasite nuclei, the immunofluorescence signal and an overlay of this image with the transmission image. Scale bar = 5 μm.

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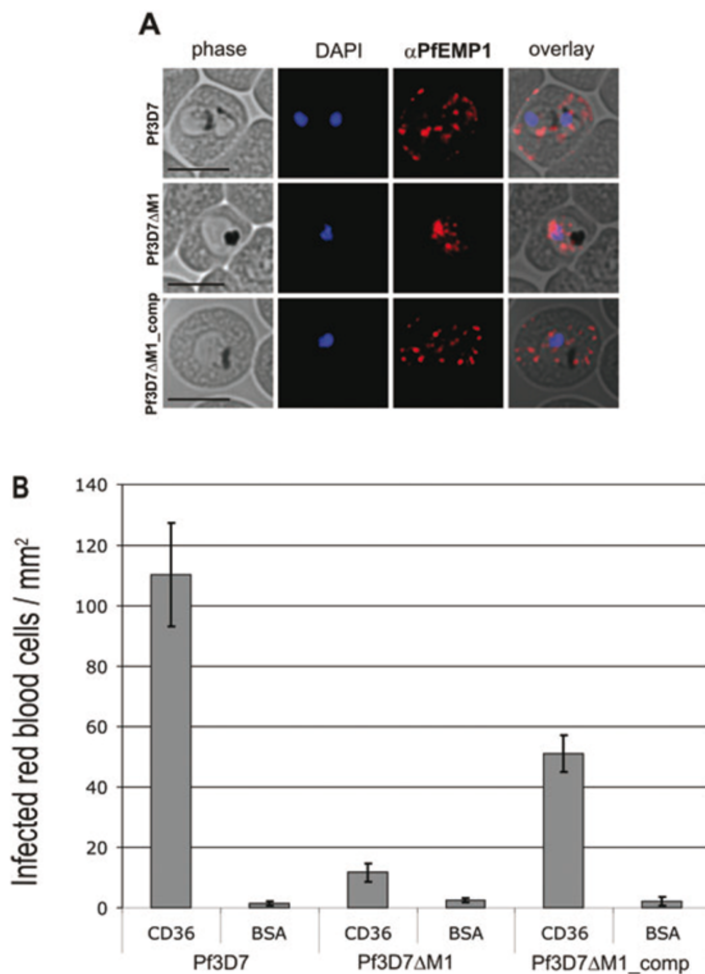

Fig. 7. Complementation of Pf3D7ΔM1 restores correct trafficking of PfEMP1 and adhesion to CD36.
A. Immunofluorescence assays of fixed RBCs infected with Pf3D7ΔM1_comp, Pf3D7ΔM1 or wild-type Pf3D7 probed with anti-PfEMP1. Each row represents a bright field image, a DAPI stain of the parasite nuclei, the immunofluorescence signal and an overlay of this image with the transmission image. Scale bar = 5 μm.
B. Bar graph depicting differential binding to CD36 of RBCs infected with Pf3D7ΔM1_comp, Pf3D7ΔM1 or wild-type Pf3D7. CD36 (or BSA as control) was immobilized onto plastic Petri dishes. Bound infected RBCs were quantified in 8 or 10 different areas (0.2 mm²). The mean value was calculated as infected RBCs per mm² and normalized to a parasitaemia of 1%.

upper row shows the typical Maurer's clefts pattern of MAHRP1 in wild-type cells, which is absent in the MAHRP1-disrupted strain Pf3D7ΔM1 (middle row). Fixed Pf3D7ΔM1_comp cells probed with anti-MAHRP1 or anti-HA tag (bottom panels) show patterns comparable to that for wild-type parasites. This is consistent with HA-MAHRP1 being correctly trafficked to the Maurer's clefts.

PfEMP1 trafficking to the host cell surface is restored in mahrp1*-complemented parasites*

To investigate if the restored presence of MAHRP1 in Pf3D7ΔM1_comp also restored the export of PfEMP1, we performed immunofluorescence assays probing with anti-PfEMP1 and comparing the phenotype of the complemented strain with that of the parental strain and the MAHRP1-disrupted Pf3D7ΔM1. Figure 7A shows that PfEMP1 is present beyond the parasites confines in discrete puncta in the RBC cytoplasm in both Pf3D7 and Pf3D7DM1_comp. This is in contrast to the labelling obtained for Pf3D7ΔM1 (middle row) where no PfEMP1 labelling is detected beyond the parasite's confines, underlining the essential function of MAHRP1 in the export of PfEMP1.

To confirm that PfEMP1 is again surface-exposed in Pf3D7ΔM1_comp-infected RBCs, we performed Western analysis of control and trypsin-treated infected RBCs (Fig. 4, lanes 5 and 6). In Pf3D7ΔM1_comp parasites, the full-length PfEMP1 (band > 250 kDa) again appears to be Triton-insoluble and is present in the sample loaded onto the gel. When the cells were pre-treated with trypsin, a

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remnant stump of about 80 kDa is observed, indicating that a part of the population of PfEMP1 is exposed at the RBC surface. The size of the N-terminal fragment is slightly different in the complemented strain compared with the parent strain, suggesting that a *var* gene switch might have occurred during the time required for development of the transfectants.

To confirm that complementation of the MAHRP1-disrupted strain with MAHRP1 not only restored the export of PfEMP1, but also one of the key functions of PfEMP1, we performed assays for binding to immobilized CD36 using bovine serum albumin (BSA) as a control. Figure 7B compares the different CD36 binding abilities of Pf3D7, Pf3D7ΔM1 and Pf3D7ΔM1_comp. The decreased CD36 binding in Pf3D7ΔM1-infected erythrocytes is partly restored in the MAHRP1-complemented strain.

Discussion

Plasmodium falciparum replicates asexually in terminally differentiated RBCs that are devoid of any organelles. The parasite resides within a PV, feeding on haemoglobin and subverting the physiology of the RBC by remodelling the host compartment. To do this, the parasite establishes an export system for proteins that are involved in nutrient uptake or in mediating cytoadherence and antigenic variation.

A number of proteins destined for the RBC membrane, such as PfEMP1, PfEMP3 and KAHRP, are trafficked via the Maurer's clefts. However, the precise role(s) of these organelles as an intermediate in the trafficking process is not yet completely understood. MAHRP1 is a Maurer's cleft-resident protein and is transcribed exclusively during early parasite stages although the protein persists throughout the asexual life cycle (Spycher *et al.*, 2003). To test the functional importance of MAHRP1, we generated a *P. falciparum* 3D7 strain which is deficient in this protein (Pf3D7ΔM1). We showed that the endogenous locus has been disrupted by a 5′ single cross-over integration of the plasmid. This disrupted the endogenous promoter which ablated protein expression as judged by Western blot analysis.

We examined whether the absence of MAHRP1 had an effect on the presence of Maurer's clefts or on the presence of other Maurer's cleft-resident proteins. Immunofluorescence microscopy showed that SBP1, REX1 and MAHRP2 were still present at the Maurer's clefts. However, PfEMP1 appeared to accumulate within the parasite (probably within the endomembrane system) and was not observed associated with the Maurer's clefts. Moreover, when the RBC membrane was permeabilized with EqtII, the Maurer's clefts became swollen and fragmented, suggesting that they are more fragile. Immunoelectron microscopy (EM) confirmed that the mutant Maurer's clefts are not labelled with anti-PfEMP1 antiserum. These data indicate that Maurer's clefts are still assembled in the absence of MAHRP1, but MAHRP1 is needed for stabilizing these structures, and for efficient delivery of PfEMP1 to the RBC surface.

It is important to note that three Maurer's cleft-resident proteins, MAHRP1, REX2 and SBP1, have several features in common. They are all expressed very early during the intra-erythrocytic cycle and no homologues have been found in other *Plasmodium* species or in other organisms. They each possess a single predicted transmembrane domain and no N-terminal ER entry signal sequence. Furthermore, the C-terminal domains of MAHRP1 and SBP1 which are oriented towards the cytoplasm are quite basic, whereas the luminal domains are more acidic. A similar dichotomy is apparent between the N- and C-terminal domains of REX2. In contrast, the cytoplasmic domain of PfEMP1 is highly acidic. Finally, none of these Maurer's cleft-resident proteins has a recognizable PEXEL/VTS motif, suggesting that they are trafficked across the PVM via a different route from that used by proteins such as KAHRP. Indeed, data obtained with MAHRP1–GFP transfectants suggest that this protein is inserted into nascent Maurer's clefts as they form (Spycher *et al.*, 2006).

We used immunofluorescence microscopy to determine whether parasite proteins are still exported in the absence of MAHRP1. While the trafficking of proteins that are exported to the RBC cytoskeleton, namely KAHRP and PfEMP3, was not affected, export of the major virulence factor PfEMP1, which is usually trafficked to the RBC surface via Maurer's clefts, was ablated. In the parent line, PfEMP1 was found in the Maurer's clefts from the early ring stage onwards. However, in the MAHRP1-deficient strain, PfEMP1 accumulates within the confines of the PVM. PfEMP1 is soluble in Triton X-100 during transit through the parasite's secretory system, but becomes Triton-insoluble when it reaches the Maurer's clefts or RBC membrane (Kriek *et al.*, 2003; Papakrivos *et al.*, 2005). In the MAHRP1 deletion strain, PfEMP1 appears to be Triton-soluble and, as a result, is lost during sample work-up for Western analysis. Proteolytic digestion of surface-exposed PfEMP1 confirmed the lack of PfEMP1 at the surface of Pf3D7ΔM1-infected RBCs. The fact that KAHRP and PfEMP3 are trafficked correctly while PfEMP1 trafficking is disrupted suggests that different modes of transport are employed for soluble cargo and PfEMP1.

To provide further evidence that MAHRP1 is essential for the export of PfEMP1 beyond the parasite's confines, we complemented Pf3D7ΔM1 with a plasmid encoding *mahrp1*. We showed by immunofluorescence microscopy and trypsin digestion studies, that in the restored presence of MAHRP1, PfEMP1 is correctly exported to the Maurer's clefts and partly exposed at the RBC surface.

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The restored surface exposure reconfers CD36 binding ability to Pf3D7ΔM1_comp-infected RBCs and makes PfEMP1 susceptible to trypsin cleavage again.

One might speculate that MAHRP1 acts as a chaperone for PfEMP1 as RAP1 has been shown to act as an escort for the rhoptry protein RAP2 (Baldi *et al.*, 2000). Alternatively, MAHRP1 might be responsible for correct insertion of PfEMP1 into nascent Maurer's clefts, for example, by recognition of the PEXEL/VTS signal motif. Recently, deletion mutants of another Maurer's cleft-resident protein, SBP1, have been generated in two separate parasite strains. In both cases, Maurer's clefts were still formed; however, there was a discrepancy in the phenotypes of the two strains with respect to trafficking of PfEMP1. When SBP1 was disrupted in the 3D7 strain, PfEMP1 was still exported to Maurer's clefts, but not further transferred to the infected RBC surface (Cooke *et al.*, 2006). The ability of infected RBCs to adhere to endothelial cell receptors was reduced in the SBP1-deficient parasites, but could be restored by complementation with SBP1 (Cooke *et al.*, 2006). By contrast, when SBP1 was disrupted in the CS2 strain, PfEMP1 trafficking was arrested at the PVM (Maier *et al.*, 2006). This is similar to the phenotype that we observe with parasites in which the MAHRP1 locus was disrupted. Thus, it is possible that MAHRP1 and SBP1 act in concert during the transport of PfEMP1 to the Maurer's clefts.

Together, these studies indicate that Maurer's cleft-resident proteins play a crucial role in the export of PfEMP1 to the surface of the infected RBC. They may have important structural roles or they may interact with cargo *en route* to the RBC membrane. It is still a matter of some debate how cargo proteins, such as KAHRP and PfEMP1, are trafficked to the Maurer's clefts and from there transferred onward to the RBC membrane. Based on 3D reconstructions of EM sections, Wickert and colleagues postulated that Maurer's clefts and the PVM form a continuous network (Wickert *et al.*, 2004). In contrast, we have reported that nascent Maurer's clefts are formed as subcompartments of the PVM, but that peripheral Maurer's clefts are independent structures that have lost their functional connection to the PVM (Spycher *et al.*, 2006).

These conflicting views of the architecture of the Maurer's clefts lead to different, although not necessarily exclusive, models for trafficking of proteins to the Maurer's clefts. If the Maurer's clefts remain connected to the PVM, soluble proteins could diffuse from the PV lumen along connecting strands to the Maurer's cleft lumen and then translocate through protein transporters into the RBC cytoplasm. Integral membrane proteins, such as PfEMP1, could transfer by lateral diffusion along the connected membrane network from the PVM to the Maurer's clefts. If, on the other hand, the Maurer's clefts are independent structures, soluble proteins could reach the RBC cytoplasm by transiting through a translocator located in the PVM. These proteins would then diffuse across the RBC cytoplasm before interacting with the cytoplasmic surface of the Maurer's clefts and eventually the RBC membrane. PfEMP1 could be incorporated into nascent Maurer's clefts before they bud from the PVM. Alternatively, PfEMP1 could be trafficked across the RBC cytoplasm in vesicles or as protein complexes and assemble at the peripheral Maurer's clefts (for reviews see Cooke *et al.*, 2004; Marti *et al.*, 2005; Lanzer *et al.*, 2006). While additional work is needed to distinguish between these different possibilities, it is clear that MAHRP1 and SBP1 play important roles as accessory proteins at various steps in these processes. In this context, it is intriguing to note that both MAHRP1 and SBP1 do not possess an obvious PEXEL motif (Marti *et al.*, 2005).

In conclusion, we have shown that loss of MAHRP1 prevents transfer of PfEMP1 to the Maurer's clefts by blocking its transfer from the parasite or PVM; this suggests that MAHRP1 might perform a chaperone function. Loss of MAHRP1 seems to change the morphology of the Maurer's clefts, suggesting that it also could play a role as a structural component. Taken together with other recent reports, the work presented here shows that Maurer's cleft-resident proteins play a very important role in trafficking of PfEMP1 to the RBC surface. Better understanding of factors that interfere with the formation or function of this compartment could lead to the development of innovative antimalarial strategies.

Experimental procedures

Parasites

Plasmodium falciparum parasites (3D7 strain) were cultured *in vitro* (Scheibel *et al.*, 1979) in RPMI medium supplemented with 0.5% AlbuMAX (Invitrogen) or 4% human serum plus 0.25% AlbuMAX. Growth synchronization at the ring stage was achieved by sorbitol lysis (Lambros and Vanderberg, 1979).

Plasmid constructs

To disrupt the *MAHRP1* gene in 3D7 parasites, flanks of the *MAHRP1* gene were cloned as potential recombination sites into the *P. falciparum* transfection plasmid *pHHT-TK* (Duraisingh *et al.*, 2002) to result in the plasmid *pTK-M1*. The 5′ flank from −957 to −260 bp of *MAHRP1* was PCR-amplified using primers 5′-atatgtatttttacttgtgaag-3′ and 5′-aacataaaaagaaaaaaaaatattccacg-3′, and cloned into *pHHT-TK* upstream of the *hdhfr* using SacII and HpaI restriction sites. The 3′ recombination region from +903 to +1508 bp was PCR-amplified using the primers 5′-aggagaaccatg tgaattccaaaaagc-3′ and 5′-gccaaacacttcatgaggagataatg-3′, and cloned into *pHHT-TK* downstream of *hdhfr* using EcoRI and SfoII restriction sites.

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To complement Pf3D7DM1 with *mahrp1*, parasites were transfected with plasmid *pBcamR_3xHA* (Ch. Flück and T. Voss, unpublished). For this, *mahrp1* was PCR-amplified using primers 5′-attaggatccatatcctaggatggcagagcaagcagc-3′ and 5′-atatgctagcattatcttttttttcttgttctaattttg-3′ and cloned upstream of an HA tag into BamHI and NheII sites of *pBcamR_3xHA*.

Plasmodium falciparum *transfection*

Plasmodium falciparum-infected RBCs (ring stage) were transfected by electroporation with 100 μg of the *pTK-M1* or *pBcam*(*3xHA*)*M1* plasmid.

pTK-M1 contains a human *DHFR* cassette and a cassette for expression of *Herpes simplex* thymidine kinase, as negative selectable marker which phosphorylates the nucleoside analogue Ganciclovir (Hoffmann-LaRoche) into the toxic Ganciclovir-triphosphate. *pTK-M1*-transfected parasites were cultured in the presence of 10 nM WR99210, a plasmodial DHFR inhibitor, for 20–30 days until viable parasites were observed in Giemsa-stained smears (Crabb and Cowman, 1996; Fidock and Wellems, 1997). Ganciclovir was added to half of the culture and the other half was taken off drug altogether for 3 weeks. Parasites treated with Ganciclovir died and the culture did not recover. Therefore, the culture taken off drug was treated with 10 nM WR99210 and kept on drug until parasites were observed (approximately 1 week, end of cycle 1). The procedure was repeated three times until a population of parasites resistant to Ganciclovir was observed. These parasites were subjected to Southern blotting analysis to detect integration events and termed Pf3D7DM1.

For complementation, Pf3D7DM1 parasites were transfected with *pBcam*(*3xHA*)*M1* containing blasticidin deaminase which converts toxin blasticidin-S to a non-toxic deaminohydroxy derivative (Mamoun *et al*., 1999). Transfected parasites were cultured in the presence of 2.5 μg ml^{-1} blasticidin-S hydrochlorid for 20 days until a stable population was established. The drug pressure was then increased to 8.5 μg ml^{-1}.

DNA extraction and Southern blotting for Pf3D7DM1 analysis

Genomic DNA was prepared by phenol/chloroform extraction of saponin-lysed, proteinase K-treated parasites as described previously (Beck, 2002). Precipitated genomic DNA was re-suspended in TE buffer. Genomic DNA (from 3D7 and 3D7ΔM1 strains) and the *pTK-M1* plasmid were digested with Mlu *1* and Tsp*R1*, separated on a 0.7% agarose gel and transferred to a nylon membrane. Southern blot hybridization was performed using standard procedures and hybridization detected by ECL (Amersham). The probe, corresponding to the 3′ flank of *MAHRP1*, was PCR-amplified from *pTK_M1* using the primers 5′-aggagaaccatgtgaattcca aaaagc-3′ and 5′-gccaaacacttcatgaggagataatg-3′.

SDS-PAGE and Western blot analysis

Synchronized trophozoite cultures (> 5% parasitemia) were saponin-lysed (0.09% final concentration) and the washed pellet was taken up in reducing SDS sample buffer (Invitrogen). Proteins were separated on a 10% or 12% bis-Tris gel (Invitrogen). Western blotting to nitrocellulose (0.45 μm, Schleicher and Schuell, Germany) was performed according to standard protocols and processed for antigen detection with a chemiluminescence system (ECL, GE Healthcare). A mouse anti-MAHRP1-C antibody (1:1000; Spycher *et al*., 2003) was used for antigen detection and the secondary antibody was horseradish peroxidase-coupled sheep anti-mouse Ig (1:1000, Chemicon). The HA tag was detected using a rat anti-HA antibody (1:1000, Roche) and a horseradish peroxidase-coupled goat anti-rat secondary antibody (1:2000, Acris).

Detection of surface expressed PfEMP1 by trypsin protection

Parasites were synchronized by sorbitol, grown to trophozoite stage (20–28 h post invasion) at a parasitemia of approximately 5% and harvested by magnet cell sorting (CS columns; Miltenyi Biotec). Infected RBCs (10^6 cells) were either treated with TPCK-treated trypsin (Sigma) 1 mg ml^{-1} or incubated in PBS for 10 min at 37°C. The reaction was stopped by addition of soybean trypsin inhibitor (1 mg ml^{-1}; Sigma). Cells were lysed on ice with Na_2HPO_4. The pellet was taken up in 2× Laemmli sample buffer. Sample was loaded onto a 5% Tris-Glycine gel and subjected to SDS-PAGE at 150 V for 90 min. The proteins were transferred onto nitrocellulose (Hybond-C Extra, Amersham Biosciences), and the blot probed with monoclonal mouse anti-ATS antibody (1:500) [generated at WEHI monoclonal lab: 1B/98–6H1-1(27)] for PfEMP1 detection. Horseradish peroxidase-conjugated anti-mouse secondary antibody (Pierce, 1:4000) and ECL detection kit (GE Healthcare) was used for visualization.

Immunofluorescence microscopy

Thin smears of infected RBCs on glass slides were fixed with acetone : methanol or 100% methanol for 10 min at −20°C and optionally permeabilized with 0.1% Triton X-100 for 10–30 min followed by a washing step. Samples were blocked for 1 h with 1% BSA in PBS, and then incubated for 1 h at room temperature with mouse anti-MAHRP1-C (Spycher *et al*., 2003; 1:200), mouse anti-SBP1 (Blisnick *et al*., 2000; 1:200), rabbit anti-REX1 (Hawthorne *et al*., 2004; 1:500), rabbit anti-MAHRP2-C (C. Spycher and HP. Beck, unpubl. data; 1:250), rabbit anti-KAHRP (Knuepfer *et al*., 2005b), rat anti-HA tag (1:100, Roche) or mouse anti-PfEMP1 1B/98–6H1-1 (1:75; Maier *et al*., 2007). Slides were washed twice and subsequently incubated with goat anti-mouse FITC (1:500, Kierkegaard KPL), goat anti-mouse cy3 (1:500, Jackson Immunological) or goat anti-rabbit cy3 (1:500, Jackson Immunological) in the presence of DAPI or Hoechst 33256 (1 μg ml^{-1}).

Electron microscopy

Parasites were harvested at the trophozoite stage by floatation on a Percoll cushion washed and fixed in 0.1 M sodium cacodylate, pH 7.4, containing 1% glutaraldehyde, 0.5%

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paraformaldehyde for 18 h. The cells were rinsed and pelleted at 1650 *g* before being post-fixed for 1 h with 1% osmium tetroxide. After washing, the cells were stained 'en-bloc' with 2% aqueous uranyl acetate and further rinsed before serial dehydration and embedding in LR-White. For immunogold EM, infected RBCs (10^8 cells, ~5% parasitemia) or Percoll-purified mature stage infected RBCs (10^8 cells) were fixed with 2% paraformaldehyde in RPMI for 15 min at 20°C, washed twice with PBS then treated with Equinatoxin II (EqtII) (2 HU) for 6 min at 20°C (Jackson *et al.*, 2007), then re-fixed with 2% paraformaldehyde in PBS for 5 min. The cells were incubated with rabbit anti-PfEMP1 cytoplasmic domain (Kriek *et al.*, 2003; 1:10), or anti-SBP1 (Blisnick *et al.*, 2000; 1:10) and 6 nm gold-conjugated protein A (Aurion), fixed in 2.5% glutaraldehyde, post-fixed with OsO_4, 'en-bloc'-stained with uranyl acetate and embedded in LR-White resin. The blocks from both experiments were sectioned (70 nm thickness), stained with uranyl acetate and lead citrate, and observed at 80 kV with a JEOL (Tokyo, Japan) 2010HC.

For the tomographic images, cells were immunolabelled with antibodies against SBP1, fixed and embedded, and then cut in thick sections (300 nm). Tomograms were acquired with a Tecnai G2 TF30 electron microscope (FEI) as described previously (Hanssen *et al.*, 2008).

CD36 binding assay

Recombinant human CD36 (125 μg ml^{-1} in PBS) or 1% BSA in PBS (control) was immobilized on a Petri dish as described previously (Voss *et al.*, 2006). Dishes were incubated in a humid chamber over night at 4°C. Non-specific binding was blocked with 1% BSA in PBS for 30 min at room temperature and washed with RPMI-Hepes. Infected erythrocytes in RPMI-Hepes and 10% human serum (5% hematocrit) were added and incubated for 30 min at 37°C. The dish was gently washed with RPMI-Hepes. Bound cells were fixed with 2% glutaraldehyde in RPMI-Hepes for 2 h and stained with 10% Giemsa for 10 min. Bound infected RBCs were quantified in 8 or 10 different 0.2 mm^2 areas. The mean value was calculated as infected RBCs per mm^2 and normalized to a parasitemia of 1%. The experiment was performed three times.

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Chapter 3

MAHRP2, an exported protein of *Plasmodium falciparum*, is an essential component of Maurer's cleft tethers

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Summary

Upon invasion into erythrocytes, the malaria parasite *Plasmodium falciparum* must refurbish the host cell. The objective of this study was to elucidate the location and function of MAHRP2 in these processes. Using immunofluorescence and immunoelectron microscopy we showed that the membrane-associated histidine-rich protein-2 (MAHRP2) is exported during this process to novel cylindrical structures in the erythrocyte cytoplasm. We hypothesize that these structures tether organelles known as Maurer's clefts to the erythrocyte skeleton. Live cell imaging of parasite transfectants expressing MAHRP2–GFP revealed both mobile and fixed populations of the tether-like structures. Differential centrifugation allowed the enrichment of these novel structures. MAHRP2 possesses neither a signal peptide nor a PEXEL motif, and sequences required for export were determined using transfectants expressing truncated MAHRP2 fragments. The first 15 amino acids and the histidine-rich N-terminal region are necessary for correct trafficking of MAHRP2 together with a predicted hydrophobic region. Solubilization studies showed that MAHRP2 is membrane associated but not membrane spanning. Several attempts to delete the *mahrp2* gene failed, indicating that the protein is essential for parasite survival.

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Introduction

Malaria is a major health problem having a huge impact on the economy and social structures in developing countries (Sachs and Malaney, 2002). The most severe and lethal form of the disease is caused by the apicomplexan parasite *Plasmodium falciparum*, resulting in close to one million deaths per year (World Health Organization, 2009). The pathology of malaria is due exclusively to the intraerythrocytic stages of the parasite's life cycle. The choice of the erythrocyte as a cell to parasitize is unusual, seen only for three apicomplexan parasites, *Plasmodium, Theileria* and *Babesia*, and two bacteria, *Anaplasma* and *Bartonella*. The malaria parasite develops within a parasitophorous vacuole (PV) inside a cell that lacks all organelles, including the machinery for protein synthesis and protein trafficking. Hence, immediately upon invasion *Plasmodium* must refurbish its host cell.

This restructuring includes the formation of flattened membrane-bound cisterna called Maurer's clefts that are thought to function as a surrogate Golgi (Atkinson *et al.*, 1988; Elford *et al.*, 1997; Lanzer *et al.*, 2006; Tilley and Hanssen, 2008). The clefts play an important role in the transport to the erythrocyte surface of the major virulence protein, *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP1; Bhattacharjee *et al.*, 2008). The N-terminal domain of *Pf*EMP1 is exposed on the surface of infected erythrocytes and mediates binding to vascular endothelial receptors in a process termed cytoadherence. This is responsible for sequestration of infected erythrocytes in the microvasculature of different organs (Pasloske *et al.*, 1993; Baruch *et al.*, 1995; 1996; Voss *et al.*, 2006). Other exported proteins transiently associate with Maurer's clefts before they relocate to their final destinations, including the knob-associated histidine-rich protein, *P. falciparum* erythrocyte membrane protein-3 and the multi-gene family subtelomeric variable open reading frame (KAHRP, *Pf*EMP3, STEVOR) (Wickham *et al.*, 2001; Kriek *et al.*, 2003; Knuepfer *et al.*, 2005a,b; Przyborski *et al.*, 2005; Blythe *et al.*, 2008).

Several integral membrane proteins are known to be residents of Maurer's clefts and some of them play an

essential role in the trafficking of *Pf*EMP1. Parasites carrying gene deletions of the Maurer's cleft resident proteins, membrane-associated histidine-rich protein-1 (MAHRP1) and skeleton-binding protein-1 (SBP1) fail to present *Pf*EMP1 on the surface and do not adhere to the endothelial receptor CD36 (Cooke *et al.*, 2006; Maier *et al.*, 2007; Spycher *et al.*, 2008). Many Maurer's cleft resident proteins are exclusively expressed at the early stages of the parasite's life cycle, but the mechanism for genesis of the Maurer's clefts is still debated.

Recent studies have suggested that the Maurer's clefts are independent compartments derived from PV membrane extensions (Spycher *et al.*, 2006; Hanssen *et al.*, 2010). The name is in fact a misnomer; Maurer's clefts often lie close to the RBC surface membrane but do not connect directly to the host cell surface. Indeed, recent electron tomographic analyses have revealed the presence of tubular elements that appear to tether the closed Maurer's cleft bodies to the erythrocyte membrane (Hanssen *et al.*, 2008a; 2010); however, the composition and origin of the tethers is not known.

Recently, the process for export of parasite proteins to the RBC compartment has become clearer. Most exported proteins carry a pentameric signal sequence (RxLxE/Q), downstream from the hydrophobic N-terminal signal sequence, called the *Plasmodium* export element (PEXEL) or host targeting (HT) signal (Hiller *et al.*, 2004; Marti *et al.*, 2004). This motif is cleaved off in the endoplasmic reticulum by an aspartic protease, Plasmepsin V (Boddey *et al.*, 2010; Russo *et al.*, 2010), and the newly formed N-terminus is acetylated (Chang *et al.*, 2008). An ATP-powered translocon transfers these PEXEL positive proteins across the PV membrane (de Koning-Ward and Gilson, 2009).

However, several exported proteins, including MAHRP1 and SBP1, lack both a PEXEL motif and a classical signal sequence (see Maier *et al.*, 2009 for review). For both these proteins the transmembrane domain and the second half of the N-terminal domain are sufficient for export and transfer to the Maurer's clefts (Spycher *et al.*, 2006; Saridaki *et al.*, 2009). Another PEXEL-negative protein is ring-exported protein-1 (REX1), which is translocated across the PV membrane as a soluble protein, which then docks at the Maurer's clefts (Dixon *et al.*, 2008). The N-terminal hydrophobic stretch plus an additional 10 amino acids is sufficient to direct export of this protein, while a coiled-coil region in the C-terminal domain is needed for binding to the Maurer's clefts (Dixon *et al.*, 2008). In contrast, the first 10 amino acids of the N-terminal domain and the transmembrane domain are sufficient to target ring-exported protein-2 (REX2) to Maurer's clefts (Haase *et al.*, 2009). Interestingly, the first 10 amino acids of REX2 can be substituted with the first 10 amino acids of SBP1 or MAHRP1, suggesting a similar means of transport.

Here, we describe a new signal sequence- and PEXEL-negative exported protein with some similarities to MAHRP1. This protein, which we refer to as membrane-associated histidine-rich protein-2 (MAHRP2) (PlasmoDB accession number PF13-0276), was found in a transcription analysis of early transcribed genes (Spielmann and Beck, 2000). We have now analysed the sequence requirements for correct export and show that it is exported to the previously described Maurer's cleft tethers (Hanssen *et al.*, 2008a). We were able to partly purify these unusual cellular structures by ultracentrifugation and prove the presence of MAHRP2 in these structures. This is the first protein to be identified that is exclusively associated with these structures.

Results

MAHRP2 is a small exported protein

MAHRP2 was first reported in a study using suppression subtractive hybridization to identify *P. falciparum* (3D7 strain) genes exclusively transcribed at early ring stages of the parasite (Spielmann and Beck, 2000). *mahrp2* (PF13-0276) lies on chromosome 13 and encodes a protein of 137 amino acids with a predicted pI of 6.86. The protein sequence includes a histidine-rich (non-repetitive) N-terminal domain and a central region with 15 hydrophobic amino acids (Fig. 1A). The protein lacks a classical signal sequence and has no PEXEL motif. MAHRP2 has some similarities to MAHRP1 (MAL13P1.413), which has a histidine-rich domain (based on DHGH repeats) at the C-terminus. MAHRP2 is completely conserved and has no orthologous gene in any other *Plasmodium* species.

Fig. 1. MAHRP2 is exported to the cytoplasm of the erythrocyte and associates with punctate structures.
A. Structure of MAHRP2. The N-terminal domain (amino acids 1–72) is indicated in yellow, the hydrophobic region used for domain swapping (amino acids 73–92) in red, and the C-terminal domain (amino acids 93–137) in blue. Italized *H* mark the histidine rich region of the N-terminal domain.
B. Dual label immunofluorescence microscopy of formaldehyde or acetone/methanol-fixed 3D7 smears using antibodies recognizing MAHRP2 (green) and anti-MAHRP1, anti-SBP1 and anti-PfEMP1 (red), or (last panel) anti-MAHRP1 (green) and anti-SBP1 (red). Scale bar = 5 µm.
C. Scatter plots showing the results from colocalization analyses of the respective markers in the entire image stack (anti-MAHRP2 and anti-MAHRP1) and single images (anti-MAHRP1 and anti-SBP1).
D. Western blot analysis of hypotonically lysed cells at different stages *P. falciparum* strain 3D7. Early ring stage (2–9 h post infection (hpi)), trophozoite (22–30 hpi) and late trophozoite/schizont stage (39–47 hpi). The blot was probed with rabbit anti-MAHRP2 serum.

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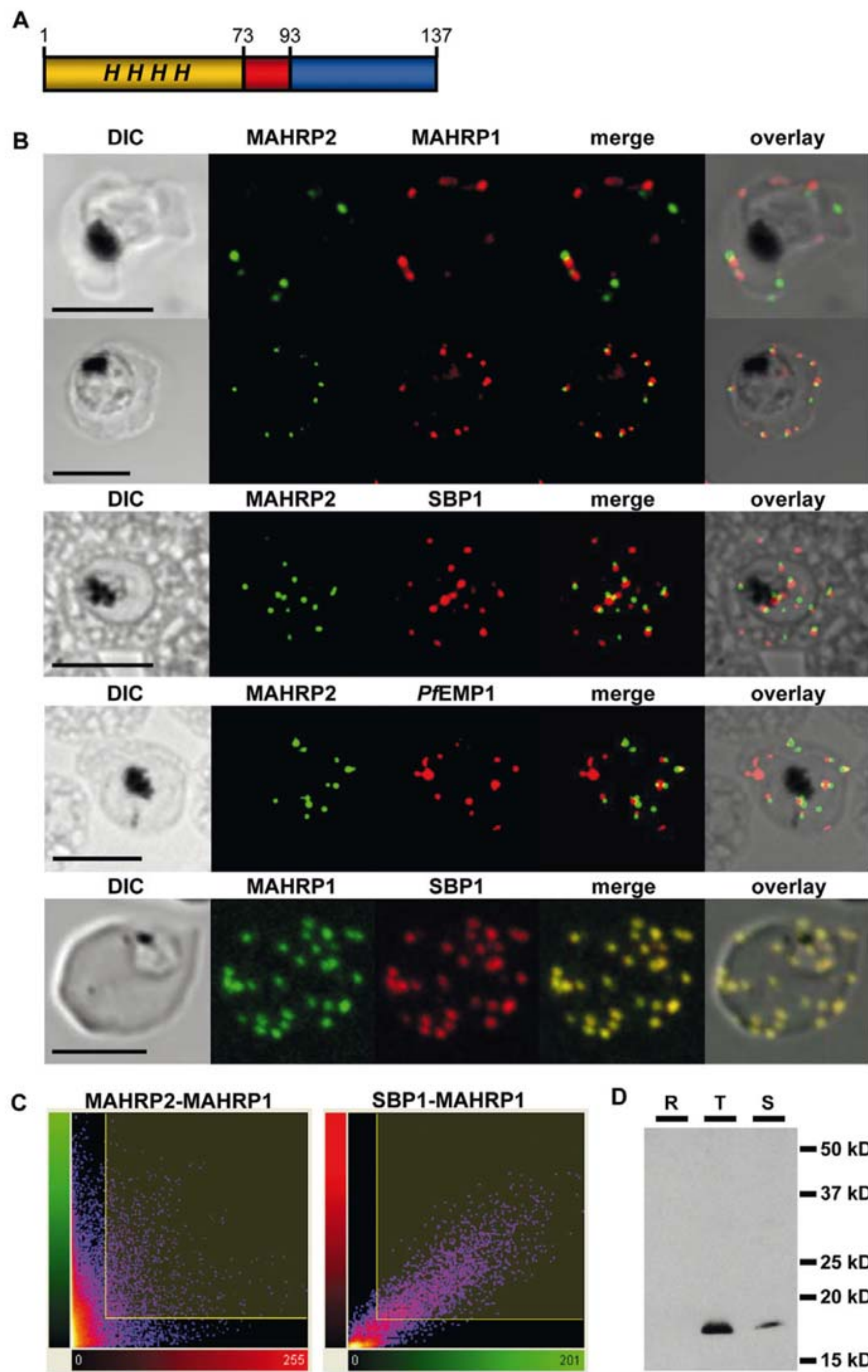

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We made several attempts to genetically disrupt the *mahrp2* gene using protocols that have previously been used successfully to generate MAHRP1 deletion mutants (Spycher *et al.*, 2008). However, these efforts were not successful, suggesting that MAHRP2 is essential for parasite survival.

Subcellular location of MAHRP2

We raised antisera against the C-terminal domain of MAHRP2 in rabbits. The affinity purified antibodies recognized a band of approximately 17 kD (Fig. 1D) in good agreement with the predicted molecular mass (15.8 kDa). Our previous transcription analysis revealed high MAHRP2 mRNA levels in early ring stage parasites (Spielmann and Beck, 2000); however, we were not able to detect MAHRP2 protein in ring stage parasites by Western blot analysis (Fig. 1D) or by immunofluorescence microscopy (data not shown). The protein is maximally expressed in the trophozoite stage (Fig. 1D). The anti-MAHRP2 antibodies gave no signal on preparations from uninfected erythrocytes (data not shown).

In mature stage parasites the antibodies reacted with punctate structures in the erythrocyte cytoplasm (Fig. 1B) in a pattern that is somewhat reminiscent of Maurer's cleft staining. We undertook dual labelling of cells with a range of Maurer's cleft-associated proteins. Interestingly, we found no colocalization of MAHRP2 with MAHRP1, SBP1 or *Pf*EMP1; however, the signals were often in close proximity (Fig. 1B and C, Fig. S1). This suggests that MAHRP2 is associated with a separate compartment of the exomembrane system of *P. falciparum*.

We generated transfectants expressing MAHRP2 C-terminally fused to green fluorescent protein (GFP). The full-length *mahrp2-GFP* chimera was inserted into the transfection vector pARL1a (Crabb *et al.*, 2004) under the control of the *PfCRT* 5′ region. *PfCRT* 5′ is a moderate promoter that drives maximal expression in the late ring stages (Bozdech *et al.*, 2003), which is similar to the expression profile for MAHRP2 (Spielmann and Beck, 2000). When the lysates were probed with antibodies recognizing GFP, no reactivity was observed in uninfected RBCs or in the 3D7 parent parasites on Western blots (data not shown), while an approximately 47 kD protein was observed in MAHRP2-GFP transfectants (Fig. 2A).

We examined the location of MAHRP2–GFP in formaldehyde-fixed trophozoite stage infected RBCs that were co-labelled with the membrane probe, BODIPY-ceramide (Adisa *et al.*, 2003) (Fig. 2B). BODIPY-ceramide strongly labels the membranes within the parasite and also enables visualization of the erythrocyte and parasite-derived membranous structures (including Maurer's clefts) found in the erythrocyte cytoplasm. MAHRP2–GFP shows a similar punctate pattern to endogenous MAHRP2 (Fig. 2B). The GFP signal appeared to be separate from the BODIPY-ceramide-labelled structures in the erythrocyte cytoplasm although often closely adjacent to. We found that treatment of very early ring stage parasites for 20 h with brefeldin A (Lippincott-Schwartz *et al.*, 1989) caused trapping of the MAHRP2–GFP within the parasite (data not shown). This indicates that MAHRP2 is trafficked within the parasite via the classical secretory pathway.

We examined immunofluorescence micrographs of MAHRP2–GFP transfectants that were colabelled with antibodies recognizing REX1 (Fig. 2C). As for endogenous MAHRP2 the signals were usually in very close proximity but did not overlap. To determine the location MAHRP2 at the ultrastructural level we used antibodies against MAHRP2 or GFP to label 3D7- or MAHRP2–GFP transfectant-infected RBCs that had been permeabilized using the pore-forming toxin, Equinatoxin II [EqtII (Hanssen *et al.*, 2008a)]. In transmission electron micrographs the Maurer's clefts were observed as single slender cisternae with an electron-dense coat and an electron-lucent lumen (Fig. 3). The Maurer's cleft bodies were not recognized by either immune reagent; however, electron-dense tubes or filaments adjacent to Maurer's clefts were strongly decorated with gold particles (Figs 3 and S2). These cylindrical structures have been previously described (Hanssen *et al.*, 2008a; 2010) and are thought to be tethers that link the Maurer's clefts to either the erythrocyte membrane or the PV membrane.

In some of the parasites within a culture that has been recently transfected with the episomally encoded MAHRP2–GFP, the transgene may be very highly expressed due the presence of construct concatamers; these highly expressing cells are lost with time in culture (Crabb *et al.*, 2004). Interestingly, in electron micrographs of recent transfectants we observed stacks of these tethers in some of the cells (Fig. S2). While this stacking of tethers was unusual the data suggest that MAHRP2 may be the major component of the tethers and that overexpression of this component may be sufficient to drive the production of additional tethers.

Solubility characteristics of MAHRP2

The host cell membranes of erythrocytes infected with trophozoite stage parasites were selectively disrupted with saponin to release haemoglobin, and then the parasites were lysed hypotonically to release soluble proteins. Peripheral membrane proteins were extracted from the insoluble pellet with sodium carbonate and integral membrane proteins with Triton X-100. The remaining material was solubilized with SDS (Haase *et al.*, 2009). GAPDH, a soluble protein in the parasite cytoplasm, was found in the

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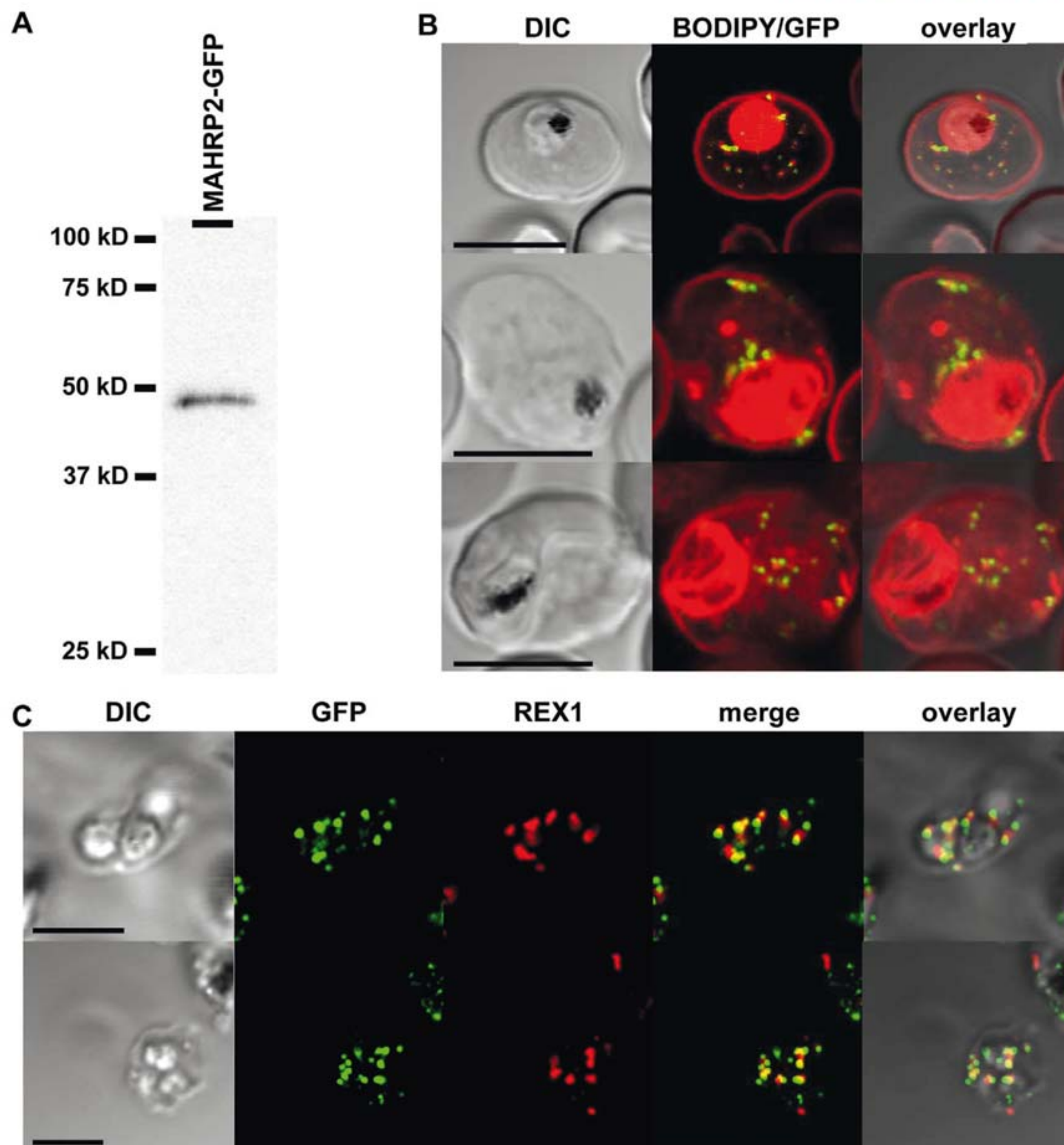

Fig. 2. MAHRP2–GFP labels punctate structures adjacent to Maurer's clefts.
A. Western blot analysis of saponin-insoluble material from erythrocytes infected with MAHRP2–GFP transfectants probed with mouse anti-GFP antibodies.
B. Fluorescence microscopy of erythrocytes infected with MAHRP2–GFP transfectants (green). Samples were fluorescently labelled with the lipid probe, BODIPY-ceramide (red), and fixed with formaldehyde. DIC image are shown at left and in the overlays.
C. Dual labelled immunofluorescence microscopy of formaldehyde-fixed and EqtII-permeabilized MAHRP2- GFP transfectants. Parasites were labelled with mouse anti-GFP antibody (green) and rabbit anti-REX1 antibody (red). Scale bar = 5 µm.

supernatant after hypotonic lysis, while MAHRP1, a proven integral membrane protein of Maurer's clefts (Spycher *et al.*, 2003; 2006), was only extracted by treatment with Triton X-100 (Fig. 4A).

Somewhat surprisingly, MAHRP2 was extracted with sodium carbonate. This indicates that MAHRP2 is peripherally associated with membranes and it is unlike that it is an integral membrane protein. To investigate this further

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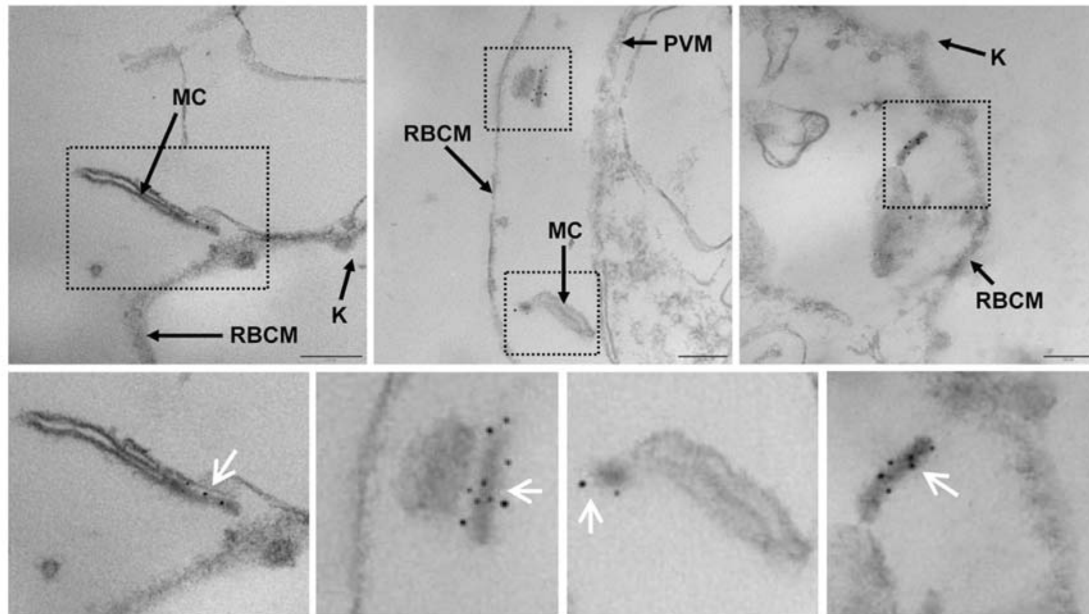

Fig. 3. MAHRP2 is associated with electron-dense tubules in the cytoplasm of infected erythrocytes.
Thin sections through EqtII-permeabilized erythrocytes infected with 3D7 parasites. The sections were probed with rabbit anti-MAHRP2 antibody, and subsequently decorated with protein A-gold conjugate (6 nm). Maurer's clefts (MC) have an electron-lucent lumen and an electron-dense coat. The structures labelled by MAHRP2 are slender electron-dense tubules (white arrows) that are often associated with the Maurer's clefts. K, Knobs; RBCM, red blood cell membrane; PVM, PV membrane. Bars are 200 nm.

we performed a Triton X-114 extraction of saponin pellets of parasitized erythrocytes. Triton X-114 forms a homogeneous phase at 0°C but separates into aqueous and detergent phases above 20°C. Hydrophilic proteins are concentrated in the aqueous phase, whereas integral membrane proteins are recovered in the detergent phase (Bordier, 1981). Proteins with lipid modifications such as myristoylation are also recovered to some extent in the detergent phase (Martens *et al.*, 2004). MAHRP2 was analysed in trophozoite stage (25–34 hpi) and late trophozoite/schizont stage (34–43 hpi) parasites and was found exclusively in the aqueous phase (Fig. 4B). No MAHRP2 was found in ring stage parasites (3–12 hpi). MAHRP1 was recovered, at least in part, in detergent phase as expected for an integral membrane protein (Fig. 4B, lower panel).

Requirements for export of MAHRP2

MAHRP2 lacks a classical signal sequence and an obvious PEXEL motif. To elucidate the sequence requirements for export we transfected parasites with constructs expressing truncated versions of *mahrp2* fused to a GFP tag, and analysed the location of the fusion proteins. Each of the chimeric proteins migrated on SDS-PAGE with apparent molecular masses close to those predicted (Fig. 5A). In the MAHRP2-N-terminal domain fusion the level of expression was very low and a longer exposure of the blot was required (right hand panel).

When MAHRP2 N-terminal or C-terminal domains alone were fused to GFP the chimera remained inside the parasite (Fig. 6, panels 2 and 5). Similarly, a chimera comprising the hydrophobic and C-terminal domains was not exported (Fig. 6, panel 4). In contrast, a chimera of the hydrophobic and N-terminal domains was translocated to punctuate structures in the erythrocyte cytoplasm (Fig. 6, panel 3). Thus, the N-terminal region seems to carry essential information for correct trafficking.

To investigate this further we generated additional constructs (Fig. 6). Deletion of the first 12 amino acids of the N-terminal domain abolished transport completely (Fig. 6, panel 6). Replacing the first 15 amino acids with those of MAHRP1 did not rescue the export (Fig. 6, panel 10). Deletion of a region preceding the hydrophobic domain (amino acids 51–72) had no effect on export, whereas deletion of amino acids 25–53 of the N-terminal domain containing the histidine-rich part also abolished export of the chimeric protein (Fig. 6, panels 7, 8). Substitution of amino acids 25–53 with amino acids 25–53 of MAHRP1, which does not include a known motif or histidine-rich

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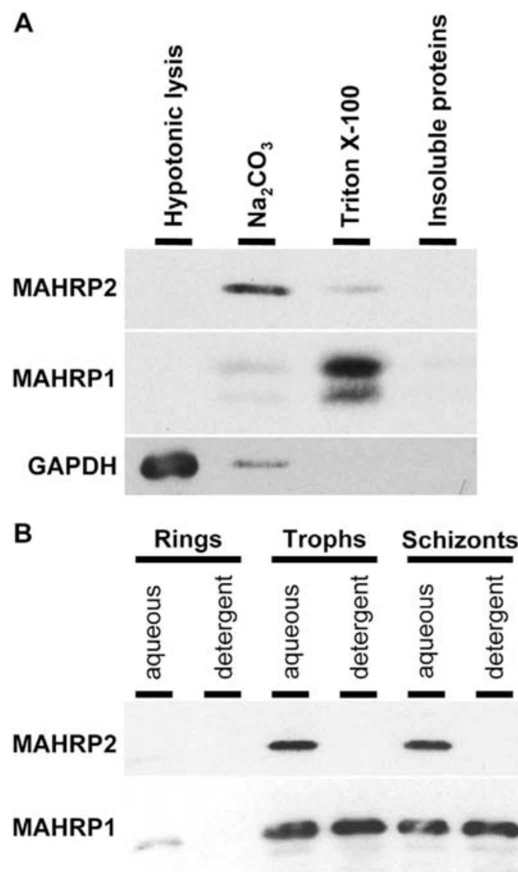

Fig. 4. MAHRP2 is a peripheral membrane protein.
A. Western blot analysis of fractions from solubility assays of erythrocytes infected with wild-type 3D7 parasites. Blots were probed with rabbit anti-MAHRP2 antibodies (panel 1), with rabbit anti-MAHRP1 antibodies (panel 2), and with mouse monoclonal antibodies against GAPDH (panel 3). Lanes represent soluble proteins after hypotonic lysis, Na_2CO_3-releasable peripheral membrane proteins, Triton X-100-soluble integral membrane proteins, and the remaining insoluble fraction.
B. Western blots of Triton X-114-fractionated protein extracts from 3D7 parasites in different stages were probed with rabbit anti-MAHRP2 antibodies (panel 1) and rabbit anti-MAHRP1 antibody (panel 2). MAHRP2 was found only in the aqueous phase.

repeats, did not re-establish correct transport (Fig. 6, last panel). A PEXEL-like motif, QHLGE, is located at position 21–25; however, mutation of leucine and glutamic acid to lysine and alanine had no effect on export (Fig. 6, panel 9). Interestingly, replacement of the MAHRP2 hydrophobic domain with the hydrophobic domain of the integral membrane protein MAHRP1 did neither change the localization of the chimeric protein nor the solubility characteristics (Fig. 7A and B).

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Isolation of tethers by differential centrifugation

Live imaging of MAHRP2–GFP expressing parasites indicates that there were at least two populations of tethers. Some of the GFP-labelled structures were very mobile within the erythrocyte cytoplasm while others were immobile (Fig. S3). This is in agreement with a recent whole-cell electron tomography study (Hanssen *et al.*, 2010), showing that some of the 'tethers' have one or both ends unattached. A differential high speed centrifugation approach was used to enrich for the free population of connecting structures. Erythrocytes infected with parasites expressing MAHRP2–GFP were lysed hypotonically and subjected to eight sequential centrifugation steps at increasing *g* forces. The pellets from each step were probed by Western blotting and MAHRP2 was found in fractions up to 5000 *g*, and in the 80 000 *g* pellet (Fig. 8A). Similar findings were observed with wild-type 3D7 parasites (Fig. 8B) and with parasites transfected with a MAHRP2 construct possessing an HA-tag (data not shown). By contrast, MAHRP1 was only detected in the low speed pellet (Fig. 8B).

The low speed pellets are likely to contain organelles, such as the Maurer's clefts and attached tethers that remain associated with the erythrocyte or parasite membrane. By contrast the 80 000 *g* pellet could contain the free population of tethers. We examined the 80 000 *g* pellet from wild-type parasites by transmission electron microscopy and we were able to identify tubular structures of about 100–200 nm in length and approximately 30 nm in width. Immuno-gold labelling confirmed the presence of MAHRP2 in these structures (Fig. 8C).

Discussion

During its erythrocytic cycle *P. falciparum* invades a cell devoid of all organelles and of protein synthesis and trafficking machinery. The parasite remodels its host cell early after invasion exporting a range of proteins across two membranes and into the erythrocyte cytoplasm. Integral membrane proteins are also exported; some of these are destined to sites in membrane structures that are elaborated in the host cell cytoplasm by the parasite, while others, such as *Pf*EMP1, are eventually inserted into the erythrocyte membrane. The protein trafficking pathway is quite complex, apparently completely novel and still not fully understood; however, it is clear that the Maurer's clefts play an important role in the translocation of proteins to the surface of the erythrocyte (Tilley and Hanssen, 2008).

Many exported proteins possess a conserved PEXEL/VTS motif (Hiller *et al.*, 2004; Marti *et al.*, 2004); however, a significant number of PEXEL-negative exported proteins have also been described, including MAHRP1

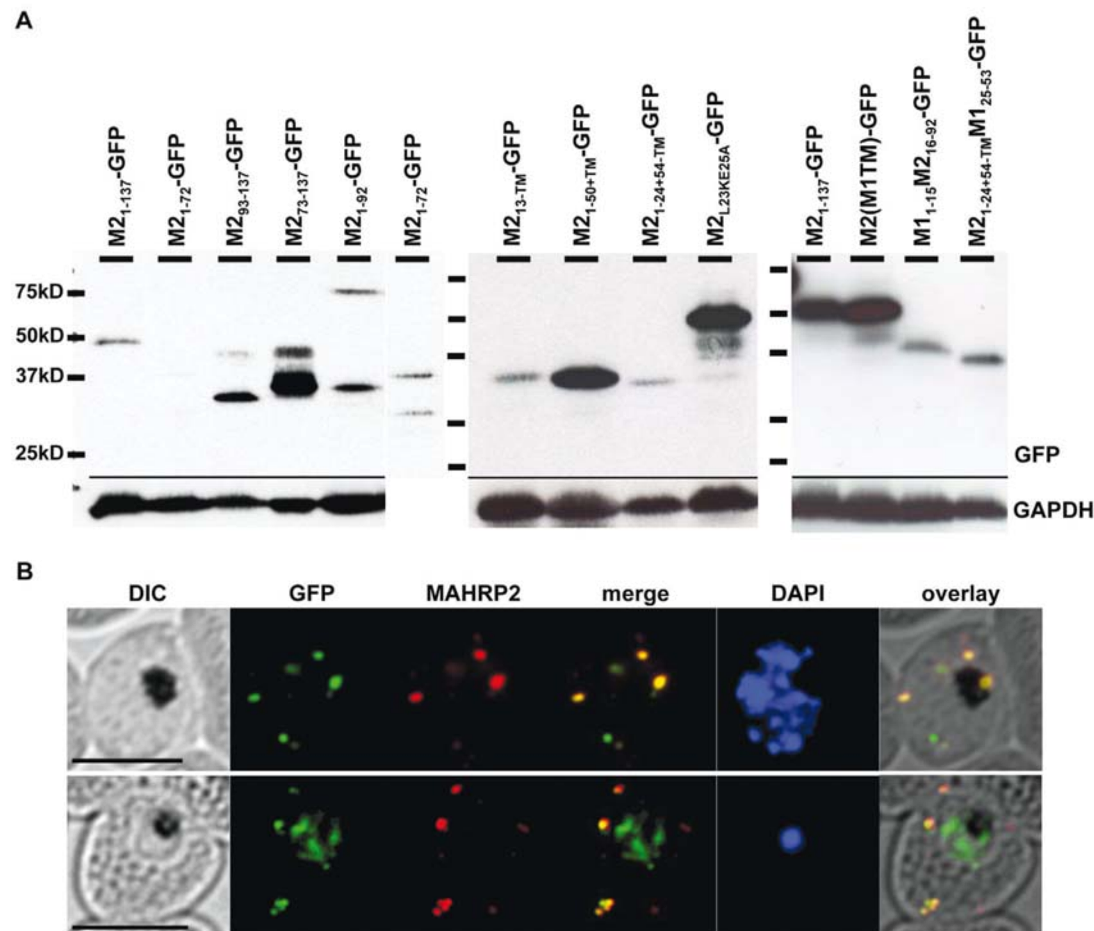

Fig. 5. Truncated MAHRP2 is correctly exported.
A. Western blot analysis of saponin-insoluble material from 3D7 parasites transfected with all truncated or mutated MAHRP2–GFP constructs. The blot was probed with mouse anti-GFP antibody. The M2-NT–GFP lane at the right side of the left blot represents a longer exposure time.
B. Dual label immunofluorescence microscopy of acetone/methanol-fixed smears of erythrocytes infected with parasites expressing $M2_{1-50+TM}$–GFP. Antibodies used were mouse anti-GFP (green) and rabbit anti-MAHRP2 (red) which only recognizes the C-terminal domain of endogenous MAHRP2. Scale bar = 5 μm

(Spycher *et al.*, 2003; 2006; 2008), SBP1 (Cooke *et al.*, 2006; Maier *et al.*, 2007; Saridaki *et al.*, 2009), REX1 (Hawthorne *et al.*, 2004; Spielmann *et al.*, 2006a) and REX2 (Haase *et al.*, 2009). *Pf*EMP1 also has a non-canonical PEXEL motif and the trafficking pathway for this important virulence protein is not clear, although MAHRP1 (Spycher *et al.*, 2008) and SBP1 (Cooke *et al.*, 2006; Maier *et al.*, 2007) have been shown to be essential for the efficient transport of *Pf*EMP1 to the erythrocyte surface.

A number of the PEXEL-negative exported proteins are expressed in the ring stage and may play a role in establishing the exomembrane system. We identified another early transcribed PEXEL-negative protein, MAHRP2 (Spielmann and Beck, 2000). MAHRP2 has some similarities in structure to MAHRP1, in that it is small and histidine-rich with a central hydrophobic domain. Despite their similarities the export-mediating sequences, as well as the location and physical organization of MAHRP1 and 2, were found to be different. In the case of MAHRP2, deletion of the first 12 amino acids completely abolished export, similarly as reported when the first 10 residues were deleted from REX2 (Haase *et al.*, 2009). In contrast, truncations of up to 74 amino acids from the N-terminus has little effect on the export of the MAHRP1 (Spycher *et al.*, 2006). Replacement of the N-terminal region of

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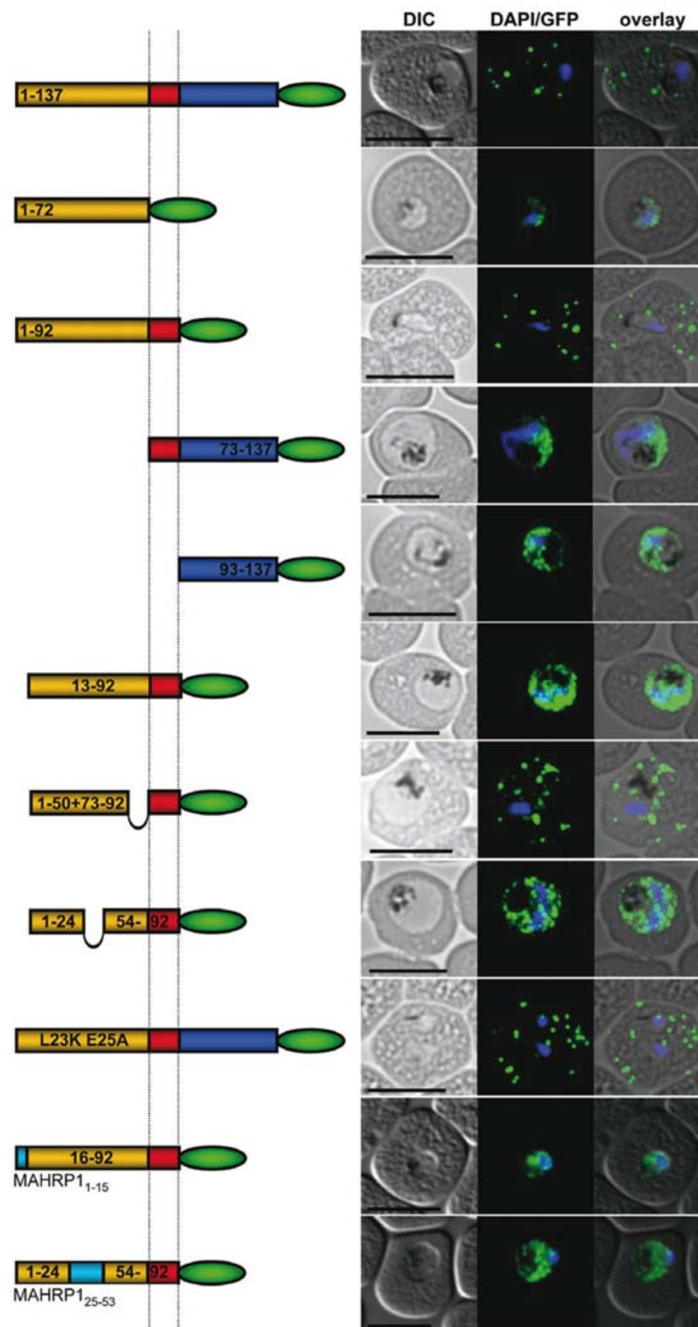

Fig. 6. The N-terminal domain and the hydrophobic domain are important for the export of MAHRP2.
Immunofluorescence microscopy of erythrocytes infected with *P. falciparum* 3D7 episomally expressing full-length and truncated or mutated forms of MAHRP2 fused to GFP. Chimeric MAHRP2 was visualized with mouse anti-GFP antibodies. The N-terminal domain is depicted in yellow, the C-terminal domain in blue, and the predicted transmembrane domain in red. Images from left to right: DIC, DAPI and GFP fluorescence, and overlay. Sequence replacements are shown in light blue.

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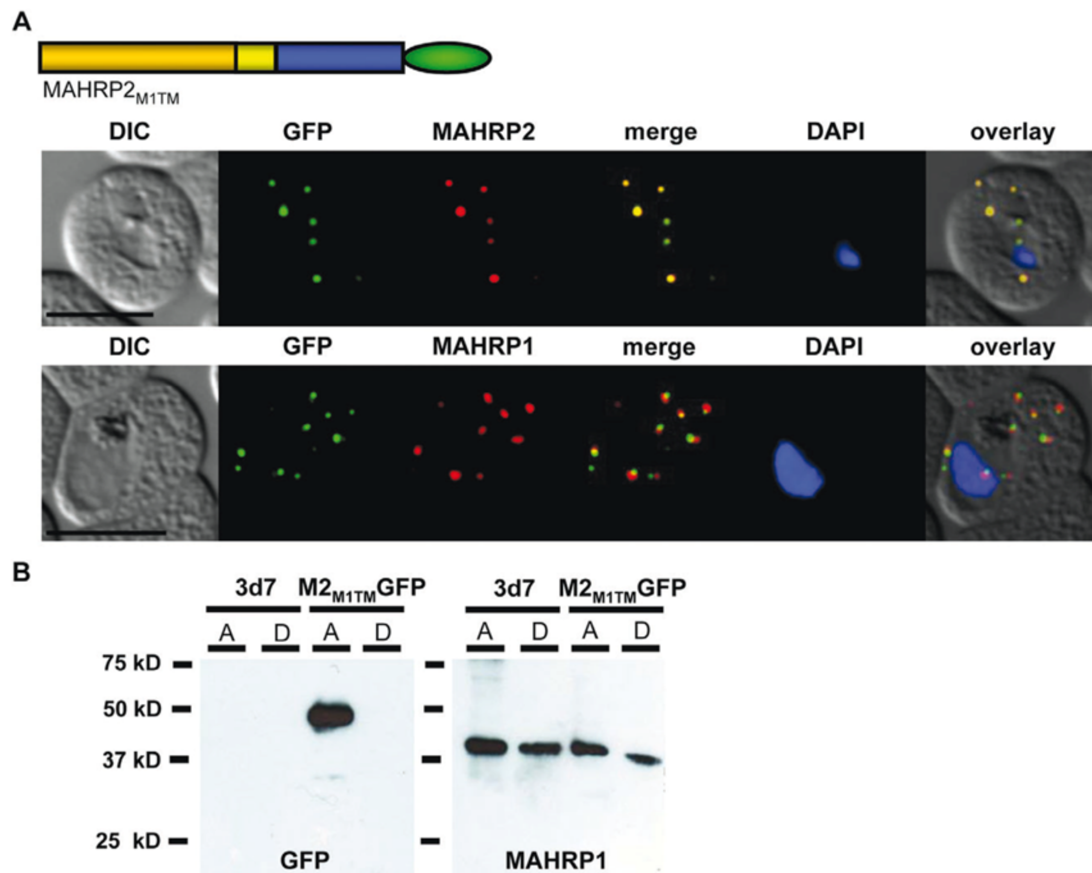

Fig. 7. Replacing the hydrophobic domain of MAHRP2 with the transmembrane domain of MAHRP1 does not change its location. For the MAHRP2(M1TM)GFP (A) transfectants dual labelling was performed with anti-GFP antibodies (green) and anti-MAHRP2 antibodies (red, top row), and anti-MAHRP1 antibodies (red, bottom row). (B) Western blots of Triton X-114-fractionated protein extracts from 3D7 and MAHRP2(M1TM)GFP expressing parasites were probed with mouse anti-GFP antibodies (panel 1) and rabbit anti-MAHRP1 antibody (panel 2). MAHRP2(M1TM)GFP was found only in the aqueous phase.

REX2 with the equivalent region from MAHRP1 was reported to rescue REX2 export (Haase *et al.*, 2009), but did not rescue MAHRP2 export.

MAHRP2 possesses an amino acid sequence (LxE), which could resemble a truncated PEXEL motif (Chang *et al.*, 2008) at position 23–25 similar to that found at amino acid position 5–7 in REX2 (Haase *et al.*, 2009). Mutation of both amino acids had no effect on the export of MAHRP2 in contrast to REX2 where mutation of the glutamic acid resulted in export deficiency. This clearly suggests that at least for MAHRP2 this motif plays no role in export.

Deletion of the histidine-rich region of the N-terminus (amino acids 25–53) also abolished the export. By contrast, the C-terminal histidine-rich repeats of the MAHRP1 were found not to be needed for correct trafficking (Spycher *et al.*, 2008). Replacing the histidine-rich sequence of MAHRP2 with the equivalent region for the N-terminal domain of MAHRP1 did not rescue the export of MAHRP2.

Thus, correct transport of MAHRP2 at least requires the first 15 amino acids, the histidine-rich domain from residues 25–53, and the hydrophobic domain. For SBP1 it was shown that the transmembrane domain directs entry into the secretory pathway, while additional domains affect the net charge distribution, which in turn affects correct loading into the Maurer's clefts (Saridaki *et al.*, 2009). It is possible that correct MAHRP2 trafficking is dependent on correct charge distribution or on correct folding of the protein to present a particular domain for recognition by the PEXEL translocon or by a carrier/chaperone protein. We showed that the trafficking of

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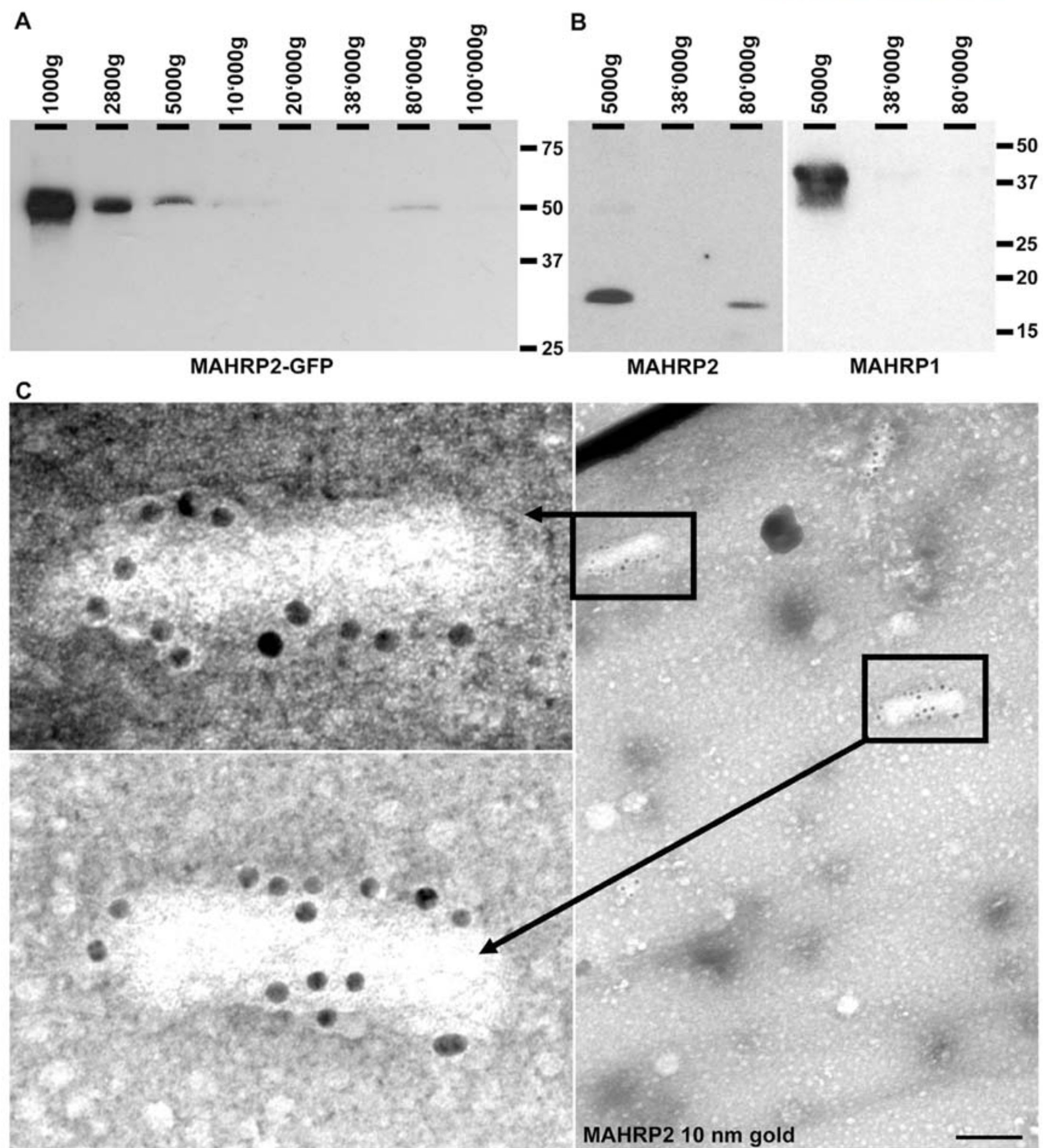

Fig. 8. MAHRP2 labelled structures can be enriched by differential centrifugation.
A. Differential centrifugation pellets of hypotonically lysed erythrocytes with parasites expressing MAHRP2–GFP were analysed by Western blot and probed with mouse anti-GFP antibodies.
B. Pellets from wild-type 3D7 parasites were probed with rabbit anti-MAHRP2 and MAHRP1 antibodies.
C. Pellets from the 80 000 *g* fraction were mounted on a grid, fixed, and probed with rabbit anti-MAHRP2 antibodies and subsequently visualized with goat anti-rabbit-gold conjugated antibodies (10 nm). Sizes were estimated to be 100–200 nm in length and 30–50 nm in width.

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MAHRP2 is brefeldin A-sensitive as has been shown for REX2 (Haase *et al.*, 2009) and for a range of PEXEL-containing exported proteins (Khattab and Klinkert, 2006; Chang *et al.*, 2008). This suggests that MAHRP2 is trafficked through the ER and suggests that the hydrophobic domain (amino acids 74–93) acts as recessed ER entry signal. Uncleaved internal signal sequences are found in some proteins of higher eukaryotes, most notably ovalbumin (Tabe *et al.*, 1984).

Solubility studies clearly show that MAHRP2 behaves like a peripheral membrane protein in contrast to MAHRP1, which clearly behaves as integral membrane protein. When the hydrophobic domain of MAHRP2 was replaced with the MAHRP1 transmembrane domain, the chimera was still trafficked to the tethers and the solubility characteristics also remained the same as for the endogenous protein (Fig. 7). This may suggest that MAHRP2 adopts a 3D conformation that favours a monotopic 'hairpin' insertion of the protein into membranes as a peripheral rather than a transmembrane domain. This conformation would likely expose both N- and C-terminal regions of MAHRP2 to the erythrocyte cytoplasm, and this might be important for the trafficking and function of the protein. Protein–protein interactions mediated by histidine-rich domains have already been demonstrated in *P. falciparum*. Deletion of two histidine-rich parts of KAHRP abolished the ability to bind to *Pf*EMP1 and to the erythrocyte membrane skeleton (Waller *et al.*, 1999). MAHRP2 may be involved in similar interactions.

Protein–protein interaction involving the histidine rich domain may also play an important role during trafficking through the ER. There is evidence in other systems for the assembly of exported soluble proteins into protein complexes with transmembrane proteins in the lumen of the ER prior to secretion. For example, the soluble subunit β2-microglobulin and an 8–10 residue peptide ligand are assembled with the glycosylated transmembrane heavy chain forming an MHC class I heterotrimeric molecule prior to transport to the cell surface (Donaldson and Williams, 2009).

We were able to show that MAHRP2 is exported to punctate structures in the erythrocyte cytoplasm that are clearly distinct from the Maurer's clefts. Indeed, electron microscopy revealed that MAHRP2 is associated with structures known as tethers (Hanssen *et al.*, 2008a; 2010). These tethers are electron-dense tubular structures that are often located in close proximity to Maurer's cleft bodies and appear to play a role in connecting the Maurer's clefts to other membranes. Such tethering functions have been suggested for *Pf*EMP3 (Waterkeyn *et al.*, 2000) and it remains to be tested whether *Pf*EMP3 colocalizes with MAHRP2. A recent whole-cell electron tomography study showed that apart from the Maurer's cleft-associated tethers there is another population of similar structures with one or both ends unattached (Hanssen *et al.*, 2010).

In live MAHRP2–GFP transfected parasites some of the labelled structures appeared to be fixed while others were moving rapidly within the cytoplasm of the infected erythrocyte. We have previously reported that in early stage MAHRP1–GFP transfectants, some Maurer's cleft bodies are freely mobile in the erythrocyte cytoplasm but become fixed in later stage parasites (Spycher *et al.*, 2006). This suggests that the Maurer's clefts system comprises modular components (i.e. bodies and tethers, with the tethers apparently in excess) and that these components can be assembled and disassembled at different positions in the erythrocyte cytoplasm at different stages of development.

We were able to enrich a preparation of the 'free' tethers by differential centrifugation and showed that the isolated tethers are heavily decorated with immuno-gold when incubated with antibodies against MAHRP2. The estimated size of these tethers was 100–200 nm in length and 30–50 nm in width which corresponds to tomographic reconstruction data of a 30 nm diameter for these tubules (Hanssen *et al.*, 2010).

Hanssen *et al.* (2010) showed that SBP1 is partly associated with the tether structures but is more concentrated on the Maurer's clefts, while MAHRP1 is exclusively associated with the Maurer's cleft bodies. MAHRP2 is the first protein that localizes exclusively to these tubular structures, and it is of interest to note that in some MAHRP2–GFP overexpressing parasites bundles of tubules were observed suggesting that MAHRP2 is the main component of the tethers.

It is important to note that all attempts to date to genetically ablate *mahrp2* have failed, indicating its importance for survival of the parasite, although it is possible to target the locus for genetic modifications (S. Rusch, E. Pachlatko, H.P. Beck, unpublished data). By contrast, deletion of MAHRP1 (Spycher *et al.*, 2008), SBP1 (Cooke *et al.*, 2006; Maier *et al.*, 2007) and REX1 (Dixon *et al.*, 2008) alters Maurer's cleft morphology and affects *Pf*EMP1 trafficking but does not affect parasite growth *in vitro*. Our data indicate that MAHRP2 is a protein that plays a very important role in parasite development. The correct assembly of the Maurer's clefts at the erythrocyte membrane may be required for the delivery of proteins that are needed for uptake of nutrients, for the maintenance of ion gradients or for the remodelling and stabilization of the RBC membrane during intraerythrocytic development. The generation of conditional knockdown parasites and further proteomic analysis might shed more light in future on the role of these new structures of *P. falciparum*.

In conclusion, we describe here the first protein localizing exclusively to the recently described tubular structures in the cytoplasm of the erythrocyte that connect

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Maurer's clefts to the erythrocyte membrane or the PV membrane. The sequence requirements for the export of MAHRP2 are different from proteins that are exported to Maurer's clefts, suggesting that conformational signals rather than simple sequence motifs may be involved in directing the export of this PEXEL-negative protein. Furthermore, the ability to enrich these structures makes them accessible to further research and functional analyses. Gaining more insight into the processes of host cell modification by the parasite might lead to novel intervention strategies and improvement in controlling this disastrous disease.

Experimental procedures

Cell culture

The *P. falciparum* 3D7 strain was cultured at 5% haematocrit as described (Trager and Jenson, 1978), using RPMI medium supplemented with 0.5% Albumax (Dorn *et al.*, 1995). Parasites were synchronized with 5% sorbitol (Lambros and Vanderberg, 1979). For total parasite extracts, infected erythrocytes from a 10 ml culture (5% haematocrit, 5–10% parasitemia) were lysed on ice using 0.03% saponin, washed three times in PBS and resuspended in Laemmli sample buffer. Infected erythrocytes from a 30 ml culture were also hypotonically lysed with 5 mM Na_2HPO_4, for 5 min on ice and after washing with PBS resuspended in Laemmli sample buffer.

Generation of MAHRP1C and MAHRP2C rabbit polyclonal antibodies

Rabbit antisera were produced by Pacific Immunology, Ramona CA, USA. In short, for each protein two New Zealand white rabbits were immunized four times with 250 µg of recombinantly expressed C-terminal domains of MAHRP1 or MAHRP2 fused to a GST tag (MAHRP1C-GST/ MAHRP2C-GST) and Freund's adjuvant. Animals were sacrificed after 13 weeks. Sera samples (20 ml) were affinity purified using MAHRP1C-GST and MAHRP2C-GST coupled to HiTrap NHS-activated HP columns (GE-Healthcare, 1 ml). The columns were washed with 50 ml PBS, bound IgG was eluted with 0.1 M glycine, pH 2.5, and the buffer was exchanged to PBS using HiTrap Desalting Columns (GE Healthcare). Purified antibodies were stored at –80°C until further use.

Solubility analysis

An aliquot (10 ml) of a culture of infected erythrocytes (trophozoite stage 3D7) was saponin lysed, then the pellet was treated with 200 µl of 5 mM Tris-HCl, pH 8, containing complete protease inhibitor cocktail (Roche) and frozen at –80°C. After thawing, the lysate was centrifuged at 16 000 *g* for 10 min and the supernatant analysed as the soluble protein fraction. The pellet was resuspended in 200 µl 0.1 M Na_2CO_3, and kept on ice for 30 min to extract peripheral membrane proteins. After centrifugation the supernatant was analysed as the carbonate extract. Integral membrane proteins were extracted from the pellet with 1% Triton X-100 on ice for 30 min. After centrifugation the supernatant was analysed as the integral membrane protein fraction, and the pellet was extracted at room temperature with 200 µl 4% SDS/0.5% Triton X-114/0.5× PBS to obtain the Triton-insoluble fraction. Equivalent amounts of the samples were analysed by Western blot.

Triton X-114 partitioning analysis

An aliquot (10 ml) of parasite culture was lysed with saponin and pelleted. The pellet was resuspended in 200 µl 1% Triton X-114 in PBS containing complete protease inhibitor cocktail, on ice, for 30 min. After centrifugation for 15 min at 3000 *g*, the supernatant was collected and incubated for 5 min at room temperature, then centrifuged at 20 000 *g* for 1 min. The supernatant was analysed as the aqueous phase. The pellet was washed twice with PBS, and resuspended in the same volume as the aqueous phase, and analysed as the detergent phase. Equivalent amounts of the samples were analysed by Western blotting (Bordier, 1981; Martens *et al.*, 2004).

Plasmid construct for parasite transfection

The full-length *mahrp2* gene was PCR amplified using primers 5′-ggaatcttaagatgcagccttgtccatatg-3′ and 5′-atgggccctaatgtttgttgtgtactagtag-3′ and cloned 5′ to *gfp* into pARL1mGFPmT (preciously described (Crabb *et al.*, 2004; Spycher *et al.*, 2006) via AflII and ApaI restriction sites. Truncated constructs for trafficking studies were designed in a similar way using the PCR primers as shown in Table S1 and the restriction sites, AflII and ClaI.

Parasite transfection

Ring stage 3D7 *P. falciparum*-infected red blood cells were transfected with 100 µg plasmid DNA and cultured in the presence of 10 nM WR99210 as described (Fidock and Wellems, 1997; Rug *et al.*, 2004; Spielmann *et al.*, 2006b). Parasites expressing GFP chimeric proteins were obtained 3–4 weeks after transfection, and drug pressure was increased to 40 nM. Trophozoite stage-infected erythrocytes were harvested by flotation on a Percoll/sorbitol gradient (Aley *et al.*, 1986).

Electron microscopy

Purified trophozoite stage-infected erythrocytes were permeabilized with Equinatoxin II (EqtII) as described previously (Anderluh *et al.*, 1996; Jackson *et al.*, 2007). Briefly, infected erythrocytes were fixed in RPMI containing 2% formaldehyde, treated with EqtII, fixed again in PBS containing 2% formaldehyde and blocked with 3% bovine serum albumin (BSA) in PBS. Permeabilized cells were incubated with rabbit anti-MAHRP2 antibody at 1:20 in 3% BSA in PBS. Cells were washed and incubated with 6 nm gold-conjugated protein A

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and fixed with glutaraldehyde. Samples were post-fixed in osmium tetroxide and 'en-bloc' stained with uranyl acetate prior to embedding in LR White resin. The blocks were sectioned to a thickness of 70–80 nm, stained with lead citrate, and observed using a JEOL 2010HC at 80 kV.

For the analysis of structures in the 80 000 *g* pellets, samples were resuspended in 50–100 μl PBS, adsorbed onto Formvar-carbon-coated copper grids (300 mesh), and subsequently air dried and fixed with 2% formaldehyde in PBS for 30 min. Grids were washed with PBS and blocked for 1 h with 3% BSA in PBS. For immuno-electron microscopy, grids were incubated with rabbit anti-MAHRP2 antibody diluted 1:7 in 0.3% BSA in PBS, washed, and incubated with goat anti-rabbit antibody conjugated to 10 nm gold at 1:6 in 0.3% BSA in PBS, and washed. The grids were fixed in 2% glutaraldehyde in PBS for 30 min, washed in water and air-dried. Specimens were negatively stained with uranyl acetate (saturated solution in water) for 30–60 s at room temperature (Hemphill and Croft, 1997). Specimens were viewed using a Phillips 400 TEM operating at 80 kV.

Fluorescence microscopy

Labelling of membranes in MAHRP2–GFP transfectants. BODIPY-TR-ceramide (Molecular Probes) was used to label MAHRP2–GFP transfectants as described previously (Adisa *et al.*, 2003). Briefly, parasitized red blood cells were resuspended in complete medium, incubated in the presence of 1 μM BODIPY-TR-ceramide at 37°C for 24 h, and washed three times in incomplete medium. Cells were fixed in complete medium containing 2% formaldehyde for 20 min, and after washing, mounted on concanavalin A-coated coverslips.

Indirect immunofluorescence. Infected red blood cells were fixed with complete medium containing 2% formaldehyde, treated with EqtII, again fixed in PBS containing 2% formaldehyde and blocked with 3% BSA in PBS. Permeabilized cells were incubated with rabbit anti-MAHRP2 antibody, mouse anti-MAHRP1 (Spycher *et al.*, 2003), rabbit anti-REX1 (Hanssen *et al.*, 2008b), or mouse anti-GFP (Roche) at 1:20 in PBS 3% BSA. Cells were washed and incubated with FITC-conjugated goat anti-rabbit (Kierkegaard KPL) or Alexa Fluor 568 (Invitrogen) conjugated goat anti-mouse antisera, respectively, washed again, and mounted on concanavalin A-coated coverslips.

Alternatively, smears of infected red blood cells were fixed in 60% methanol and 40% acetone for 2 min at −20°C, air-dried and blocked with 1% BSA in PBS. After incubation with primary antibodies rabbit anti-MAHRP1 serum (1:500), rabbit anti-MAHRP2 serum (1:100), mouse anti-SBP1 (1:100) (Blisnick *et al.*, 2000), mouse anti-*Pf*EMP1 (1:50) (Maier *et al.*, 2007) or mouse anti-GFP (Roche, 1:100), slides were washed in 0.05% Tween-20 in PBS, and incubated with Texas Red (Invitrogen, 1:200) or Cy3 (Jackson Immuno Research, 1:500) conjugated goat anti-mouse and Alexa Fluor 488 (Invitrogen, 1:200) conjugated goat anti-rabbit antibodies. Vectashield Hard Set (Vector Laboratories) containing DAPI was added and covered by a glass coverslip. Images were obtained using a Leica DM 5000B fluorescence microscope. All images were analysed with ImageJ software (http://rsb.info.nih.gov/ij). Quantitative analysis of colocalization was done with the Imaris software suite (Bitplane, Zurich, Switzerland).

Live cell imaging. MAHRP2–GFP transfectants were mounted on concanavalin A-coated coverslips, mounted in a temperature controlled chamber and images were collected using a Zeiss LSM 510 confocal microscope as described elsewhere (Abu Bakar *et al.*, 2010).

Western blot analyses

A saponin-lysed parasite pellet was resuspended in Laemmli sample buffer, separated on a 12.5% or 15% acrylamide gel, and blotted to nitrocellulose (Hybond-C extra; GE Healthcare) for 1.5 h using a Trans-Blot semi-dry electroblotter (Bio-Rad). The nitrocellulose membrane was blocked in 8% skim milk, 0.1% Tween in Tris-buffer. The antibodies used were: rabbit anti-MAHRP2 (1:1000), rabbit (1:5000) or monoclonal mouse (1:1000) anti-MAHRP1, monoclonal mouse anti-GFP (Roche, 1:1000), horseradish peroxidase-conjugated goat antimouse (Pierce, 1:20 000) and goat anti-rabbit (Acris, 1:5000) IgG.

Differential centrifugation

MAHRP2–GFP transfectants (30 ml cultures) were lysed hypotonically on ice for 5 min with 6.5 ml 5 mM Na_2HPO_4, containing complete protease inhibitor cocktail (Roche). The lysate was centrifuged for 10 min and the pellets collected at consecutive centrifugation steps at 1000 *g*, 2800 *g*, 5000 *g*, 10 000 *g* and 20 000 *g* in an Eppendorf tabletop centrifuge at 4°C. The supernatant was then subjected to consecutive centrifugation steps at 38 000 *g* for 30 min, 80 000 *g* for 1 h, and 100 000 *g* for 1 h, using a swing out rotor (TST28.38) in a Centrikon T-2070 ultracentrifuge (Kontron). Each pellet was washed in PBS, resuspended in Laemmli sample buffer and analysed by Western blot. Erythrocytes infected with wild-type 3D7 parasites were lysed as above and subjected to consecutive centrifugation steps at 5000 *g*, 38 000 *g* and 80 000 *g*.

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Chapter 4

Elaboration of the export pathway of the PEXEL-negative exported protein MAHRP2 and a functional analysis

Data in preparation for manuscript

Introduction

As illustrated in Chapter 3 the Membrane Associated Histidine-Rich Protein 2 (MAHRP2) is a small protein involved in host cell refurbishment which is exported to the electron-dense tubular structures called tethers that seem to attach Maurer's clefts to the erythrocyte membrane (Pachlatko *et al.*, 2010). MAHRP2 is the only protein known so far that specifically localizes to these structures. The protein carries a central hydrophobic stretch that is needed for export but which does not function as a transmembrane domain as MAHRP2 is not an integral but a peripheral membrane protein. For export the N-terminus comprises additional information as the deletion of the first 15 amino acids or of the central N-terminal histidine-rich stretch led to export failure. The C-terminal domain did not have any influence on transport. We wanted to further elucidate how MAHRP2 is trafficked to tethers in the cytosol of the erythrocyte and what role the protein and tethers play in the parasite.

Because replacement of the hydrophobic domain of MAHRP2 with the transmembrane domain of MAHRP1 did not change solubility characteristics (Pachlatko *et al*., 2010) we wanted to test now whether the hydrophobic domain could in turn act as a transmembrane domain in a different context. For this we substituted the MAHRP1 transmembrane domain with the MAHRP2 hydrophobic stretch. To show that MAHRP2 depends on the classical secretory pathway through the ER we performed brefeldin A assays and generated MAHRP1- and MAHRP2-GFP fusions with an N-terminal signal peptide of the Merozoite Surface Protein 1 thereby forcing the chimeric protein into the ER. Sequence analysis of the N-terminal domain revealed at position 16 to 33 a helix with amphipathic character and a twilight zone myristoylation site for glycine 16. GFP fusion constructs with mutation of glycine 16 to alanine were generated to see a possible effect on export or localization.

As nothing is known about the function of tether structures except from descriptive electron microscopy depicting them as connection of Maurer's clefts to the red blood cell membrane (Hanssen *et al.*, 2010, Hanssen *et al.*, 2008), we

wanted to identify other tether proteins through pull down experiments with recombinant MAHRP2 and by mass spectrometric analyses of the enriched tether fraction obtained from differential centrifugation. Candidates were examined by investigating their localization in immunofluorescence assays either by the use of antibodies or through C-terminal fusion with an HA-, GFP- or TY-tag.

Because all attempts failed to delete the *mahrp2* gene we made use of the recently developed conditional knock out system in which a destabilizing domain (DD-FKBP) is fused to the protein of interest (Armstrong & Goldberg, 2007, Banaszynski *et al.*, 2006, Dvorin *et al.*, 2010, Herm-Gotz *et al.*, 2007, Russo *et al.*, 2009). The fusion protein is degraded by default through the proteasomic pathway and is stable only in the presence of the ligand Shield-1. This system has not been used before for exported proteins but it has been shown to work for cytoplasmic proteins of the parasite (Armstrong & Goldberg, 2007, Dvorin *et al.*, 2010, Russo *et al.*, 2009). We replaced the 3' end of *mahrp2* with the destabilizing domain fused to a *myc* tag and analyzed protein level regulation and phenotypic changes.

Here, we show that the hydrophobic domain of MAHRP2 functions as a transmembrane domain in the context of MAHRP1 and that MAHRP2 is transported through the ER. Although MAHRP2 is not an integral membrane protein, membrane association seems to be absolutely essential for the export of MAHRP2. We describe potential export mechanisms for MAHRP1 and MAHRP2 as well as various possible interacting partners of MAHRP2. We are, in addition, able to knock down the MAHRP2 protein levels almost completely.

Methods

Cell culture

The *P. falciparum* 3D7 strain was cultured at 5% hematocrit as described (Trager & Jenson, 1978) using RPMI medium supplemented with 0.5% Albumax (Dorn *et al.*, 1995). Parasites were synchronized with 5% sorbitol (Lambros & Vanderberg, 1979). For total parasite extracts infected erythrocytes from a 10 ml culture (5% hematocrit, 5-10% parasitemia) were lysed for 10 minutes on ice using 0.03% saponin, washed 3 times in PBS and resuspended in Laemmli sample buffer.

Brefeldin A assay

Brefeldin A (BFA) assays were performed to check for classical protein secretion as BFA leads to the redistribution of secreted proteins to the endoplasmic reticulum. BFA (Sigma) dissolved in 100% ethanol was added to cultures of erythrocytes infected with ring stage parasites to a final concentration of 5µg/mL. Control cultures were incubated in the presence of equivalent amounts of 100% ethanol. After 18 hours infected erythrocytes were fixed for immunofluorescence.

Triton X-114 partitioning analysis

To test solubility characteristics of chimeric proteins Triton X-114 extractions were carried out. Triton X-114 separates into two phases at room temperature, an aqueous phase containing soluble proteins, and a detergent phase where integral membrane proteins are recovered. An aliquot (10 ml) of parasite culture was lysed with saponin and pelleted. The pellet was resuspended in 200 µl 1% Triton X-114 in PBS containing complete protease inhibitor cocktail (Roche), on ice, for 30 minutes. After centrifugation for 15 minutes at 3000 *g*, the supernatant was collected and incubated for 5 minutes at room temperature, then centrifuged at 20,000 *g* for 1 minute. The supernatant was analyzed as aqueous phase. The pellet was washed twice with PBS, and resuspended in the same volume as the aqueous phase, and analyzed as detergent phase. Equivalent amounts of the samples were analyzed by Western blotting (Bordier, 1981, Martens *et al.*, 2004).

Plasmid constructs for parasite transfection

$M1_{M2TM}$, $M2_{G16A}$, *SP-M2*, *SP-M2ΔTM*, *SP-M1*, *SP-M1ΔTM*, and *PF08_0137* were PCR amplified using primers listed in Supplementary Table 1 and cloned 5’ to *gfp* into

pARL1mGFPmT (preciously described (Crabb *et al.*, 2004, Pachlatko *et al.*, 2010, Spycher *et al.*, 2006)) via AflII and ClaI restriction sites. PCR products of *MAL13P1.237, PF07_0008*, and *actin* (PFL2215w) were cloned 5' to the *ha*-tag into pBcamR_3xHA (Spycher *et al.*, 2008) via restriction sites BamHI and NcoI or BamHI and NotI. For the 3' end GFP replacement of *actin* the last 700 bp of *actin* were amplified and cloned 5' to *gfp* into pARL1mGFPmT via NotI and AflII removing the *crt* promoter. For the 3' end HA replacement of *actin* the last 700 bp of *actin* together with the *3'ha* tag were amplified from above described plasmid *pBcamActin(3xHA)* and cloned into pARL1mGFPmT via NotI and SpeI removing the *crt* promoter and *gfp*. For the 3' end replacement of PFL0930w the last 505 bp of the gene were amplified and cloned 5' of a *ty*-tag into pHcam(2xTY) (a kind gift from A. Pulfer and T. Voss, unpublished) via PstI and BamHI removing the *cam* promoter.

The amplified *actin_intHA* was cloned into pBcamR_3xHA via restriction sites BamHI and SacI removing the 3'*ha*-tag. *mahrp2* was amplified without the ATG and cloned via BglII and AvrII upstream of the FKBP destabilizing domain and a *myc*-tag into pARL_DD_myc (a kind gift from C. Grüring and T. Spielmann, unpublished). All constructs are displayed in Supplementary Figure 1 and primers are listed in Supplementary Table 1.

Parasite transfection

Ring stage 3D7 *P. falciparum*-infected red blood cells were transfected with 100 µg plasmid DNA and cultured in the presence of 10 nM WR99210 as described (Fidock and Wellems, 1997; Rug *et al.*, 2004; Spielmann *et al.*, 2006a) or 2.5 mg/ml blasticidin-S hydrochlorid as described (Mamoun *et al.*, 1999) for 3 to 4 weeks until a stable population was established. Drug pressure of blasticidin-S hydrochlorid was then increased to 8.5 mg/ml. To generate stable 3' replacements cultures were taken off drug for 3 weeks until drug was re-administered. This drug OFF/ON cycle was repeated once or twice until integration was checked. To generate 3' replacements with the FKBP destabilizing domain parasites were cultured in the presence of 5 nM Shield1 (Armstrong & Goldberg, 2007, Banaszynski *et al.*, 2006,

Dvorin *et al.*, 2010, Herm-Gotz *et al.*, 2007) obtained from Uday Khire, Cheminpharma.

Fluorescence microscopy

Antibodies against proteins of interest or against the tags of chimeric proteins were used in immunofluorescence assays to examine their localization. Smears of infected red blood cells were fixed in 60% methanol and 40% acetone for 2 minutes at -20°C, air-dried, and blocked with 1% BSA in PBS. After incubation with primary antibodies rabbit anti-MAHRP1 serum (1:500), affinity purified rabbit anti-MAHRP2 serum (1:100), mouse anti-SBP1 (1:100) (Blisnick *et al.*, 2000), mouse anti-*Pf*EMP1 (1:50) (Maier *et al.*, 2007), mouse anti-GFP (Roche, 1:100), mouse anti-Actin (1:200) (Ding *et al.*, 2000), rabbit anti-REX1 (1:500) (Hawthorne *et al.*, 2004), rat anti-HA (Roche, 1:100), monoclonal mouse anti-TY BB2 (1:10'000) (Bastin *et al.*, 1996), or monoclonal mouse anti-HRP1 (1:200) (Taylor *et al.*, 1987) slides were washed in 0.05% Tween-20 in PBS, and incubated with Texas Red- (Invitrogen, 1:200) or Cy3- (Jackson Immuno Research, 1:500) conjugated goat anti-mouse and Alexa Fluor 488- (Invitrogen, 1:200) conjugated goat anti-rabbit antibodies. Vectashield Hard Set (Vector Laboratories) containing DAPI was added and covered by a glass cover slip. Images were obtained using a Leica DM 5000B fluorescence microscope. All images were analyzed with ImageJ software (http://rsb.info.nih.gov/ij).

Western blot analyses

A saponin lysed parasite pellet was resuspended in Laemmli sample buffer, separated on a 12.5 or 15 % acrylamide gel, and blotted to nitrocellulose (Hybond-C extra; GE Healthcare) in Tris-Glycine buffer containing methanol for 1.5 h using a Trans-Blot semi-dry electroblotter (Bio-Rad). The nitrocellulose membrane was blocked in 8% skim milk, 0.1% Tween in Tris-buffer. The antibodies used were: rabbit anti-MAHRP2 (1:1000), rabbit (1:5000) or monoclonal mouse (1:1000) anti-MAHRP1, monoclonal mouse anti-GFP (Roche, 1:1000), monoclonal mouse anti-Actin (1:2000) (Ding *et al.*, 2000), monoclonal rat anti-HA (Roche, 1:1000), monoclonal mouse anti-GAPDH (1:5000) (Daubenberger *et al.*, 2003), monoclonal mouse anti-TY (1:10'000) (Bastin *et al.*, 1996), monoclonal mouse anti-c-Myc

(ZYMED Laboratories, 1:5000), horseradish peroxidase-conjugated goat anti-mouse (Pierce, 1:20,000), and goat anti-rabbit (Acris, 1:5000) IgG.

Bioinformatic analyses

Myristoylation and amphipathic helix predictions on the MAHRP2 amino acid sequence were performed using the following websites.

http://mendel.imp.ac.at/myristate

http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html

Recombinant protein expression in *Escherichia coli*

MAHRP2C was cloned via restriction sites SmaI into the expression vector pGEX-6P-2 (Amersham Bioscience) downstream of glutathione S-transferase (GST-tag) using primers 5'-actgtacgtattgtaaatataacacc-3' and 5'-gtgtgctcttataatgtttgttgtg-3'. MAHRP2N was cloned via restriction sites BamHI and XhoI into pET-41a(+) (Novagen) downstream of a GST-tag. LB-medium was supplemented with 100 µg/ml ampicillin or 25 µg/ml kanamycin and 34 µg/ml chloramphenicol for overnight cultures, inoculated with *E. coli* (BL21 and DE3-tuner + pMico) carrying the respective expression plasmid, and incubated at 37°C shaking at 220 rpm. The overnight culture was diluted 1:100 in 400 mL fresh supplemented LB-medium and grown at 37°C shaking to an OD_{600} of 0.6 – 0.8. Protein expression was induced by adding 1 mM IPTG. Bacteria were harvested 4 hours later by centrifugation at 4°C, 4000g for 15 minutes. Bacteria pellets were frozen at -20°C until further use.

Purification of GST-tagged recombinant proteins

Frozen bacteria pellets from expression cultures were thawed on ice and resuspended in 20 mL PBS containing complete Protease Inhibitor Cocktail (Roche). After adding 100 µg/mL lysozyme and 1 µg/ml DNAse, the suspension was incubated on ice for 10 minutes. The lysate was sonicated at 50% duty cycle with five 10 second bursts with 10 second intervals in a microtip sonicator. The sonicated lysate was centrifuged for 30 minutes at 4°C and 10'000 g. The supernatant was filtered through a 0.45 µm pore size filter. GST-tagged recombinant proteins were purified with an ÄKTAprime plus (GE Healthcare) over a GSTrap™ column (GE Healthcare) according to the manufacturer's protocol. Purified proteins were stored at -80°C until further use.

Pull down experiments

To find proteins interacting with MAHRP2 pull down experiments were conducted. Cultures (150 mL) of erythrocytes infected with mixed stages of *P. falciparum* (5-10% parasitemia) were lysed hypotonically in 5 volumes of ice cold 5 mM Na_2HPO_4 and washed. The pellet of the hypotonic lysis was further incubated with 4.5 mL 0.1% Triton X-100 at 4°C rotating for 1.5h. The Triton X-100 supernatant was subsequently precleared by incubation with glutathione sepharose beads (GE Healthcare) and 50 µg GST rotating for 2 hours at 4°C. Meanwhile 50 µg of recombinant MAHRP2N-GST, MAHRP2C-GST, or GST alone were bound to glutathione beads at 4°C for 2 hours. 1 mL precleared lysate was then incubated with recombinant protein bound to glutathione beads. Beads were washed with GST lysis buffer (20 mM Tris pH8, 200 mM NaCl, 1 mM EDTA pH8, 0.5% NP40, complete protease inhibitor cocktail 1X). Washed beads were resuspended in Laemmli sample buffer and heated for 30 minutes at 95°C with vigorous vortexing every 5 minutes. Samples were separated on a gradient (4-12%, Invitrogen) acrylamide gel. Gels were stained with SimplyBlue™ Safe Stain (Invitrogen), and individual gel lanes were sliced. Gel slices were destained with 50% acetonitrile/0.1M NH_4HCO_3, dehydrated with 100% acetonitrile, dried, and subsequently prepared after standard procedures for trypsin digestion. Briefly, dried gel pieces were incubated with 12.5 ng/µl trypsin in 50 mM NH_4HCO_3 for 12 to 20 hours at 37°C. Supernatant was kept and gel pieces were further extracted with 50% acetonitrile/0.1% formic acid. The digest was dried in a speedvac centrifuge, and dried peptides were dissolved in 0.1% formic acid/2% acetonitrile prior to LC/MS analysis. LC/MS analysis was done at the Biocenter, University of Basel. Data was analysed using the available peptide libraries at PlasmoDBvs5.5.

After separation gradient gels were also stained more sensitively with SilverQuest (Invitrogen) to see differences in protein contents.

Differential centrifugation and mass spectrometry

Infected erythrocytes (30 ml cultures) were lysed hypotonically on ice for 5 minutes with 6.5 ml 5 mM Na_2HPO_4, containing complete protease inhibitor cocktail (Roche). The lysate was centrifuged for 10 minutes and the pellet

collected at 5,000 *g* in an Eppendorf tabletop centrifuge at 4°C. The supernatant was then subjected to consecutive centrifugation steps at 38,000 *g* for 30 minutes, and 80,000 *g* for 1 hour using a swing out rotor (TST28.38) in a Centrikon T-2070 ultracentrifuge (Kontron). The pellet of the 80'000 *g* fraction containing MAHRP2 labeled structures was washed in PBS, resuspended in Laemmli sample buffer without bromophenolblue and analyzed by high resolution mass spectrometry (Orbitrap) at the Proteomic Core Facility, EPFL Lausanne. Data was analysed using the available peptide libraries at PlasmoDBvs5.5.

Southern Blot

DNA from 20 mL culture with 5% parasitemia was isolated after standard protocols. The DNA was digested with EcoRI and run on a 0.7% TBE-agarose gel at 80V for 3 hours. The gel was depurinated in 0.25 M HCl for 10 minutes, washed, denatured in 0.4 M NaOH twice for 15 minutes, and neutralized in 1M Tris pH 7.5. After transfer via capillary action to a neutral nylon Hybond N membrane (GE Healthcare), the DNA was cross-linked to the membrane by baking the membrane for 1 hour at 80°C. The membrane was prehybridized with hybridization buffer (0.5 M NaPO4, 1 mM EDTA, 7% SDS, pH 7.2) and then hybridized with 10 ng/µL biotinylated *mahrp2* or *dhfr* probes diluted in hybridization buffer. The Chemiluminescent Nucleic Acid Detection Module from Pierce was used according to the manufacturer's protocol.

Results

The hydrophobic domain of MAHRP2 is a functional transmembrane domain in the right context

MAHRP2 carries a central hydrophobic stretch which was predicted to be a transmembrane domain. MAHRP2, however, does not behave as an integral membrane protein at all (Pachlatko *et al*., 2010), even though we could show that the hydrophobic stretch is needed for the export of the protein (Pachlatko *et al*., 2010). When the transmembrane domain of MAHRP1, a proven transmembrane protein, was replaced with the hydrophobic domain of the peripheral membrane protein MAHRP2 neither the localization (Fig. 1A) nor the solubility characteristics of the chimeric MAHRP1 was changed (Fig. 1B) suggesting that the MAHRP2 hydrophobic domain can function as transmembrane domain.

MAHRP2 export is brefeldin A sensitive and goes through the ER

To test if MAHRP2 export depends on the classical secretory pathway, erythrocytes infected with MAHRP2-GFP transfectants were treated with brefeldin A (BFA). BFA is a fungal metabolite that leads to the redistribution of secreted proteins to the endoplasmic reticulum (ER) (Lippincott-Schwartz *et al.*, 1989). The treatment with BFA caused an accumulation of MAHRP2-GFP in a focused perinuclear area without export to the cytosol of the erythrocyte (Fig. 2, upper panel) whereas export in mock-treated cells was normal (Fig. 2, lower panel). Since MAHRP2 does not have any classical signal sequence or a PEXEL motif, we tested whether MAHRP2 on its way to be exported, enters the ER lumen. We generated transfectants expressing C-terminal GFP-tagged MAHRP2 with or without hydrophobic stretch and with a signal peptide derived from the merozoite surface protein 1 (MSP1) at the N-termini of the fusion proteins and thus forced the proteins to go into the ER lumen. The chimeric proteins were both recognized at the appropriate sizes in Western blots using antibodies against the GFP tag (Fig 3A). Full length SP-M2-GFP was exported correctly (Fig. 3B). Colocalization with MAHRP1 (Fig. 3B) showed the same pattern as MAHRP2-GFP without a signal peptide (Pachlatko *et al.* 2010). The chimera with a signal peptide but lacking the central hydrophobic stretch was accumulated at the periphery of

the parasite (Fig. 3C) indicating that the hydrophobic stretch is needed for the translocation into the cytosol of the erythrocyte. We have also looked at MAHRP1 which is exported through the ER as an integral membrane protein (Fig. 3D and E). In consistency with SP-M2-GFP, SP-M1-GFP was found exported to the Maurer's clefts (Fig. 3E, upper panel). SP-M1ΔTM-GFP on the other hand did not accumulate at the periphery of the parasite like SP-M2ΔTM-GFP but was normally exported (Fig. 3E, lower panel).

An N-terminal amphipathic helix with a twilight zone for a myristoylation site is predicted for MAHRP2

Bioinformatic analysis revealed an N-terminal helix with an amphipathic character from amino acids 16 to 33 with a twilight zone for a myristoylation site at glycine 16. *N*-myristoylation is thought to promote weak protein-membrane and protein-protein interactions and can regulate protein targeting and function (Farazi *et al.*, 2001). *N*-myristoylation is an irreversible protein modification that occurs either co-translationally following removal of the initiator methionine or post-translationally after proteolytic cleavage that reveals a 'hidden' myristoylation motif (Boutin, 1997, Farazi *et al*., 2001). For MAHRP2 it would be expected that the first 15 amino acids would be cleaved off prior to myristoylation of glycine 16. Previously we had shown that the first 15 amino acids are absolutely necessary for export of MAHRP2 (Pachlatko *et al*., 2010) and thus speculated that myristoylation could occur on residue 16. Interestingly, the 18 amino acids following glycine 16 are predicted to form an amphipathic helix, whereby glycine 16 lies in the centre of the nonpolar side of the wheel (Fig. 4A). To test whether glycine 16 must be myristoylated for correct translocation of MAHRP2, we generated transfectants expressing MAHRP2-GFP with glycine 16 mutated to Alanine ($M2_{G16A}$-GFP) (Fig. 4). No differences to the wild type were observed by immunofluorescence (Fig. 4B) nor were the solubility characteristics changed (Fig. 4C).

Potential interaction partners of MAHRP2

To learn more about the function of MAHRP2 we tried to identify proteins interacting with MAHRP2. In one approach, we recombinantly expressed both the MAHRP2 N- and C-terminal domains without the hydrophobic stretch fused to a

GST-tag in *E. coli* and coupled the recombinant proteins to glutathione sepharose beads. We pulled down potential protein interaction partners from parasite lysate and candidates were separated on an acrylamide gel (Fig. 5). The gel lanes were sliced and candidate proteins identified by mass spectrometry. As a control we used GST only to pull down unspecifically binding proteins and subtracted these hits from the other hits. With both domains we pulled down the Ring Infected Erythrocyte Surface Antigen (PFA0110w, RESA) and the High Molecular Weight Rhoptry Protein 2 (PFI1445w) (Supplementary Table 2). Using MAHRP2C we additionally pulled down a putative RESA (PF11_0509) and with MAHRP2N we identified *P. falciparum* Actin (PFL2215w) (Supplementary Table 2). All hits were represented with a minimum of two peptides suggesting that these were true hits; however, this does not yet affirm true interaction. We further investigated localization of Actin by immunofluorescence microscopy using antibodies raised against *Dictyostelium discoideum* Actin. This antibody also detects *Plasmodium* ActinI but does not cross react with human Actin (Ding *et al*., 2000). In Western blots it only recognized a single band at the appropriate size of 41 kD in 3D7 parasites (Fig. 6A, right panel, 3D7 wt lane). By immunofluorescence microscopy we found the parasite brightly stained with the antibody (Fig 6B) and in addition, we found labelled structures in the cytosol of the erythrocyte with a typical punctate pattern of Maurer's clefts staining (Fig. 6B). Dual-labelling with antibodies against the Maurer's clefts resident Ring Exported Protein 1 (REX1) showed perfect colocalization of both proteins (Fig. 6B, lower panel). It is noteworthy that the Actin signal did not completely colocalize with the MAHRP2 signal (Fig. 6B, upper panel). This might suggest that the Actin antibody rather stains a protein at the Maurer's clefts but not at tethers.

To verify that Actin is truly exported to Maurer's clefts we created transfectants that episomally expressed Actin either fused to an HA-tag at the 3' end or with an internal HA-tag, and we generated 3' end replacements with an HA-tag. The 3' HA-tagged episomally expressed Actin was recognized in Western blots with an anti-HA antibody at around 49 kD (Fig. 6A, left panel). The chimeric protein was also detected with the antibody against Actin visible as two bands at 41 and 49 kD (Fig. 6A, right panel). By immunofluorescence microscopy with an antibody against the

HA-tag, we detected both episomally tagged Actins inside the parasite (Fig. 6C). We attempted to replace the 3' end of endogenous actin with an HA- or a GFP-tag. We successfully tagged endogenous actin with the HA tag as seen by Western blot (Fig. 6D right panel). As we did not get a signal in immunofluorescence assays the tag was apparently hidden and not accessible by the antibody. We did not achieve any integration with the GFP-tag most probably due to its bigger size (Fig. 6D left panel). Also, even though we observed a signal of the internally tagged Actin we did not have a signal in Western blot analysis which could be a sensitivity problem.

We tried to determine the proteome of the tubular structures, the tethers, in which MAHRP2 is found as resident protein, by another approach. We enriched tethers by differential high speed centrifugation of hypotonically lysed trophozoite stage infected erythrocytes as described previously (Pachlatko *et al*., 2010). The 80'000 *g* pellet containing tethers was analyzed by high resolution mass spectrometry. As this preparation was not entirely pure, because the cells were lysed hypotonically, massive contamination with ribosomal proteins was found in the analysis. Apart from MAHRP2 which was present in large amounts, 5 interesting proteins were detected namely PF07_0008 (hypothetical protein), PF08_0137 (phist c), PFL0930w (putative clathrin heavy chain), MAL13P1.237 (hypothetical conserved *Plasmodium* protein), and actin (PFL2215w). For all proteins we generated transfectants expressing tagged versions of these proteins and analyzed them by Western blotting and immunofluorescence microscopy. All the chimeric proteins run at the appropriate size on acrylamide gels as shown by western blot (PF07_0008-HA 31 kD, MAL13P1.237-HA 46 kD, PF08_0137-GFP 175 kD, Fig. 7A). As observed with all our HA-tag fusion proteins, these proteins run slightly higher than the predicted molecular masses. The 3' end of PFL0930w was replaced with a TY-tag because of its large size (236 kD, Fig. 7B). The HA-tagged version of MAL13P1.237 was not exported as antibodies against the HA-tag stained the parasite only (Fig. 7C). PF07_0008-HA and PF08_0137-GFP were exported to the erythrocyte's cytosol (Fig. 7C), and PF07_0008-HA colocalized with the Maurer's clefts resident protein MAHRP1 (Fig. 7A, first panel). The distribution of PF08_0137-GFP was rather dispersed and no perfect colocalization was found with

MAHRP1 or MAHRP2 (Fig. 7C). Genomic integration of PFL0930w in transfected parasites was confirmed by Western blot in which antibodies against the TY-tag recognized the protein. In contrast, we were not able to localize the chimeric protein in immunofluorescence assays because the anti-TY antibody cross reacted with other parasite proteins as shown by strong signals in 3D7 wild type parasites but not in uninfected erythrocytes (data not shown). This cross reactivity was not observed by Western blot as no band appeared on 3D7 wild type lysate (Fig. 7B and data not shown).

MAHRP2 can be knocked down via the FKBP destabilizing domain

We had made several unsuccessful attempts to genetically disrupt the *mahrp2* gene using protocols that have previously been used successfully to generate MAHRP1 deletion mutants (Spycher *et al*., 2008). This suggested that MAHRP2 is essential for parasite survival. To overcome this and to produce a conditional knock out parasite line, we used the FKBP destabilizing domain (DD) system to regulate the level of MAHRP2 expression (Armstrong & Goldberg, 2007, Banaszynski *et al*., 2006, Herm-Gotz *et al*., 2007). DD-fusion proteins are expected to be rapidly degraded in the absence of the ligand Shield-1 (Shld) and stabilized in its presence. We replaced the 3' end of *mahrp2* with the destabilizing domain carrying a *myc*-tag at its 3' end. Southern blot analysis revealed correct 3' end integration of the plasmid pARL_M2DD_myc into the *mahrp2* locus (Fig. 8). As control for regulation of protein expression with Shld we used transfectants expressing a YFP-DD fusion protein. Surprisingly, although we had cultured the parasites with the integrated destabilizing domain in the presence of Shld we found almost no MAHRP2 protein. By Western blot the chimeric protein MAHRP2-DD-Myc was detected with anti-Myc antibody at the predicted molecular mass of 31 kD (Fig. 9A, middle panel). However, using antibodies against the C-terminal domain of MAHRP2 we hardly detected any fusion protein (Fig. 9A right panel). The protein could only be detected as faint band if large amounts of parasite lysate were used (data no shown). Similarly, we were not able to detect the protein in immunofluorescence with either antibody (Fig. 9B, C). Thus, we deduced that very small amounts of protein were present which we only could

detect with the more sensitive anti-Myc antibody. However, this small amount seems sufficient to allow parasite survival. In addition, we were not able to regulate the level of protein expression of MAHRP2-DD-Myc by addition or removal of Shld (Fig. 9A) and obviously were unable to stabilize the fusion protein. This might indicate that the ligand is not able to bind to the destabilizing domain. In contrast, removal of Shld in YFP-DD transfectants reduced YFP protein levels by nearly 100% (Fig. 9D).

After we had shown that MAHRP2 was only present in minute amounts, we investigated whether other exported proteins were affected by this gross reduction of MAHRP2 protein levels. However, immunofluorescence microscopy revealed no obvious difference and the surface exposed *Pf*EMP1, the knob component KAHRP, and the Maurer's clefts resident proteins, MAHRP1 and SBP1, were normally exported (Fig. 10). Also, growth rates and reproduction rates of the knock down parasites did not seem to be affected by reducing MAHRP2 levels and they were not different from wild type parasites (data not shown). Further phenotypical analysis of the knock down parasites is currently ongoing.

Discussion

The most prominent and dangerous malaria parasite is *Plasmodium falciparum* infecting human erythrocytes where it undergoes multiple rounds of asexual replication which are responsible for the disease pathology. Hiding inside the mature erythrocyte the parasite is protected from host immune responses. The parasite refurbishes its host cell considerably after invasion. This process is mediated by the export of hundreds of proteins beyond the parasite's confines into the cytosol and to the surface of the erythrocyte and by formation of parasite derived membranous structures in the cytosol of the erythrocyte called Maurer's clefts. This refurbishment is crucial for parasite survival.

Current research focuses on deciphering the function of all these exported proteins (Maier *et al.*, 2008) but also on the interplay of these proteins for the survival of parasites. The mechanism of protein transport is still not fully understood. A trafficking pathway has been proposed for a subset of proteins carrying a PEXEL motif (Hiller *et al.*, 2004, Marti *et al.*, 2004). It has been suggested that these proteins are cleaved at the PEXEL site in the ER by a 'Pexelase' protease, Plasmepsin V, before they are deposited in the parasitophorous vacuole (PV) (Boddey *et al.*, 2010, Chang *et al.*, 2008, Osborne *et al.*, 2010). A chaperone containing complex has been described in the PV as *Plasmodium* translocon for exported proteins or PTEX (de Koning-Ward *et al.*, 2009). At the translocon proteins are unfolded (Gehde *et al.*, 2009) and thought to be translocated across the PV membrane (PVM) via this membrane-bound pore containing protein complex. It is unclear, however, how the processed proteins are trafficked from the ER to the PV to be recognized by the translocon. Yet, a number of PEXEL-negative exported proteins (PNEPs) have been described, such as SBP1 (Saridaki *et al.*, 2009), REX1 (Dixon *et al.*, 2008) and REX2 (Haase *et al.*, 2009), MAHRP1 (Spycher *et al*., 2006) and MAHRP2 (Pachlatko *et al*., 2010). It is completely unclear whether PNEPs are transported via the same or a completely different route to the erythrocyte cytosol.

Here, we elaborate on the trafficking pathway, interaction partners and possible function of the PEXEL-negative protein MAHRP2. We previously have shown that MAHRP2 is the only protein known so far specifically localizing to the recently

described tethers (Hanssen *et al.*, 2010, Hanssen *et al.*, 2008, Pachlatko *et al.*, 2010). Tethers are tubular structures found in the cytosol of the erythrocyte which appear to attach Maurer's clefts to the erythrocyte membrane. MAHRP2 is a peripheral membrane protein having a central hydrophobic domain and a histidine-rich region in the centre of the N-terminal domain. The hydrophobic domain, the very N-terminal first 15 amino acids, as well as the histidine-rich region comprise information for correct trafficking of the protein (Pachlatko *et al.*, 2010).

Trafficking

The central hydrophobic domain of MAHRP2 is predicted to be a transmembrane domain. Yet, we previously have shown that this domain does not function as transmembrane domain in the context of MAHRP2 but is required for export of the protein. The replacement with the transmembrane domain of the confirmed integral membrane protein MAHRP1 did not change localization or solubility characteristics of MAHRP2 (Pachlatko *et al.*, 2010). In turn, we replaced the MAHRP1 transmembrane domain with the hydrophobic region of MAHRP2 and showed that the MAHRP2 hydrophobic domain could indeed act as a real transmembrane domain in a different context. We showed that the chimeric protein was exported similarly as endogenous MAHRP1 to the Maurer's clefts and, at least partially behaved like an integral membrane protein as it was partly recovered in the detergent phase in Triton X-114 assays. Interestingly, the MAHRP1 transmembrane domain is predicted a type II TM and thus the C-terminus would face the cytosol. In contrast, the hydrophobic domain in MAHRP2 is predicted to be a type I transmembrane domain and thus substitution of the domains should lead to an inversion of the protein orientation. This would mean that the C-terminal GFP-tag of $M1_{M2TM}$-GFP would face inside the lumen of Maurer's clefts. This speculation needs further evaluation.

Lacking an N-terminal ER entry signal MAHRP2 might be guided to the ER by the hydrophobic domain acting as a recessed entry signal. This is supported by the fact that export of MAHRP2 is sensitive to brefeldin A. The transmembrane domain of the PNEP SBP1 also has been shown to direct the protein into the secretory pathway (Saridaki *et al.*, 2009) and similarly, REX2, another PNEP, has

been shown to be exported in a brefeldin A sensitive manner as well (Haase *et al.*, 2009). Uncleaved internal signal sequences are also found in some proteins of higher eukaryotes, most notably ovalbumin (Tabe *et al.*, 1984).

The hydrophobic domain alone is unlikely to be sufficient to target MAHRP2 to the lumen of the ER and thus it makes it implausible that the protein is anchored within the ER lipid bilayer upon translation. However, the internal hydrophobic domain alone may mediate targeting to the ER membrane but without an N-terminal entry signal the protein would presumably just peripherally associate with the outer membrane of the ER, probably in a hairpin-like manner. In this case, further secretion would nevertheless be brefeldin A sensitive. To further test this hypothesis, we attached the signal peptide of the merozoite surface protein 1 (MSP1) N-terminally of MAHRP2 forcing the protein to be co-translationally inserted into the ER lumen. The secretion of the chimeric protein would be blocked if MAHRP2 was secreted independently of an ER targeting, but the chimeric protein was perfectly exported similarly to the endogenous MAHRP2 indicating that entering of the ER lumen by the N-terminus does not inhibit export to tethers. Similarly, MAHRP1 with a signal peptide was perfectly exported to Maurer's clefts. This was also the case for REX2 where the signal peptide of ETRAMP10.1 was appended N-terminally and the chimeric protein was partially exported to Maurer's clefts (Haase *et al.*, 2009). To confirm that the signal peptide correctly directs the protein into the classical secretory pathway we removed the hydrophobic domain of MAHRP2 and found the chimeric protein residing in the PV as it was shown for REX2 with an ETRAMP10.1 signal peptide lacking the transmembrane domain (Haase *et al.*, 2009). It is currently being investigated whether substitution of the first 20 amino acids of the MAHRP2 N-terminus with a signal peptide would result in a rescued export because the deletion of the first 12 amino acids as well as replacement of the first 15 with amino acids of MAHRP1 abolished correct export (Pachlatko *et al.*, 2010). It might be possible that the very N-terminal amino acids of MAHRP2 already carry information for entry into the ER although this is not predictable from the charge distribution within these amino acids. Clearly, the hydrophobic domain without these N-terminal amino acids and vice versa is not sufficient information to promote export to the erythrocyte

cytosol (Pachlatko *et al*., 2010). It needs to be checked whether ER targeting of MAHRP2 with the signal peptide leads to an insertion into the ER membrane whereby the hydrophobic domain would act as a retention signal. Based on solubility studies where we found MAHRP2 not to be an integral membrane protein (Pachlatko *et al*., 2010) we do not expect that for endogenous MAHRP2. In such a case, there must be a potential protein sorting machinery at the ER or the PVM that recognizes peripheral membrane associated as well as integral MAHRP2. As the N-terminus but not the C-terminus of MAHRP2 was shown to be needed for correct trafficking, we think that such a sorting machinery would need to have access to the N-terminus to promote export. Interestingly, unlike MAHRP2 and REX2, MAHRP1 with a signal peptide but lacking the transmembrane domain was perfectly exported. This indicates that MAHRP1 is either taking a different export pathway or that a potential protein sorting machine at the ER or PVM still recognizes this chimeric protein but not the others. As this chimera is devoid of its transmembrane domain, we suggest that it is inserted into the Maurer's clefts lumen as a soluble protein (see general discussion Figure 1).

A possible situation could be that export is mediated via an interaction with other proteins, maybe even with PEXEL-positive proteins, via the N-terminal central histidine-rich region. Histidine-rich stretches have been shown to mediate protein-protein interactions. For example, the histidine-rich region of KAHRP has been shown to interact with the cytoplasmic region of *Pf*EMP1. Such a scenario would include either the formation of a protein complex that is PEXEL-independently transported possibly via vesicular transport to the erythrocyte cytosol or rather unlikely a co-export with PEXEL-positive proteins suggesting a common export pathway. Proteins of a complex with PEXEL-positive proteins all need to be unfolded prior to transport through the translocon at the PVM which makes a co-transport implausible.

It is noteworthy that the N-terminal domain of MAHRP2 contains a putative helix with amphipathic character at amino acids 16 to 33. Amphipathic helices can mediate membrane targeting whereby the non polar side of the helix is embedded in the membrane. COPI coated vesicle formation for instance, is initiated by the protein ArfI which associates with the membrane via a short

amphipathic N-terminal helix that is covalently modified with a myristoyl chain. COPII coated vesicle proteins bind to membranes also through an N-terminal amphipathic helix which is, however, not myristoylated (for review see (Pucadyil & Schmid, 2009)). Interestingly, a rather polar glycine (position 16) lies in the centre of the non polar side of the helix which is predicted as a 'twilight zone' *N*-myristoylation site. In general, myristoylation can promote membrane attachment or even protein-protein interaction (Taniguchi, 1999).

Myristoylation of glycine at position 16 would require the cleavage of 15 amino acids at the N-terminus as only N-terminal glycines can be *N*-myristoylated. Because we had previously shown that these first few amino acids are essential for protein export we tested whether they would carry a hidden signal sequence which would be cleaved off after ER entry. For this, we generated transfectants expressing MAHRP2 in which glycine 16 was mutated to alanine. This mutant cannot be myristoylated and hence should not be transported correctly. However, mutation did not impair trafficking to tethers nor were there changes in solubility characteristics. However, whether this mutation would affect protein-protein interactions has not been tested. Even if MAHRP2 is not myristoylated the amphipathic helix could mediate protein-lipid or protein-protein interaction. It has been shown that amphipathic helices can promote protein-protein interactions through hydrophobic interactions between two helices on different peptide chains. This has been shown for apolipoproteins that bind to membrane receptors or to enzymes such as lecithin-cholesterol acyltransferase (McManus *et al.*, 2000). We often find dimers of MAHRP2 in Western blots at low β-mercaptoethanol concentrations suggesting that the amphipathic helix may indeed mediate dimerization of the protein. This would be similar to the N-terminal amphipathic helix domain of the fungal immunomodulatory protein from *Ganoderma tsugae* which is responsible for dimerization and immunomodulatory activity of the protein (Lin *et al.*, 1997). It is possible that tethers are mainly composed of MAHRP2 and some lipids forming filamentous polymers via the interaction of the amphipathic helix domains.

Interaction partners

To test whether MAHRP2 is the main component of tethers or if there are other proteins involved in forming these structures we performed pull down experiments to identify proteins directly interacting with MAHRP2. In addition, we analysed the tether containing pellet from differential centrifugation experiments by mass spectrometry.

The analysis of the differential centrifugation pellet revealed several proteins. For two we confirmed their export to the erythrocyte cytosol, PF07_0008 and PF08_0137. Both encode PEXEL-negative proteins but with a hydrophobic region recessed from the N-terminus. Both proteins occur to be membrane associated because substantial amounts of these proteins were found in the pellet after saponin lysis which is devoid of soluble exported proteins. We could further show that PF07_0008 localizes to Maurer's cleft. However, it does not occur to be a structural component of Maurer's clefts because we have shown by Western blot that MAHRP1 is not present in this pellet fraction (Pachlatko *et al*., 2010). It might be speculated that PF07_0008 is found at distal edges of the clefts as linkers to the tethers. To confirm this, electron microscopy studies are needed.

PF08_0137 is clearly exported but shows a less focused localization. It is possible that it transiently associates with tethers and/or Maurer's clefts, but we cannot exclude protein contaminations.

Interestingly, we also found in the pellet the putative clathrin heavy chain, PFL0930w. Clathrin is the major protein constituent of the coat that surrounds cytoplasmic vesicles to mediate selective protein transport. Clathrin-coated vesicles mediate transport between the plasma membrane, endosome, and trans Golgi compartments (Rothman, 1994, Schekman & Orci, 1996, Schmid, 1997). Structurally, clathrin is a triskelion (three-legged) shaped protein complex that is composed of a trimer of heavy chains each bound to a single light chain. We were able to tag this protein by replacing the 3' end with a TY-tag. Unfortunately, immunofluorescence was not possible due to cross-reactivity of the anti-TY antibody with other parasite proteins and hence we were unable to proof that these vesicles are found in the cytosol of infected erythrocyte. New transfections need to be done replacing the 3' end with a different tag. It is attractive to

speculate that clathrin coated vesicles could be responsible for protein transport into the erythrocyte's cytosol and also that clathrin coated vesicles could transport protein cargo to Maurer's clefts and tethers. In this case clathrin-proteins would associate with these organelles by membrane fusion. Indeed, vesicles in the erythrocytes cytosol have been previously observed by others (Hanssen *et al.*, 2010, Hanssen *et al.*, 2008, Kriek *et al.*, 2003, Taraschi *et al.*, 2003, Wickert *et al.*, 2003) although a function has not been assessed for these vesicles.

*Pf*ActinI was pulled down with recombinant MAHRP2 from parasite lysate. Actin is not considered to be an exported protein because it lacks all common signal sequences or export motifs. Using an antibody specific against actin of *Apicomplexan* parasites we not only detected *Plasmodium* actin within the confines of the parasite but also residing at Maurer's clefts. This has been observed previously using a different antibody (Lazarus *et al.*, 2008). In support of this, *Pf*Actin was also found in the differential centrifugation pellet. It is important to note, that infected cells were lysed hypotonically and the present actin might represent intraparasitic actin filaments which could have been spun down at a similar speed. Further evidence for actin export is coming from a recent study analysing the parasitophorous vacuolar (PV) proteome (Nyalwidhe J.O., Azimzadeh O., Przyborski J.M., Pachlatko E. *et al.*, submitted). Among others, several proteins without signal sequence or PEXEL motif were identified to reside in the PV including actin. This demonstrates that these proteins are secreted and are either at their final destination or are further exported to the erythrocyte cytosol.

We were unable to show that tagged versions of *Pf*Actin are exported to the erythrocyte cytosol. This could be a sensitivity problem because the ratio between tagged actin and intraparasitic non-exported actin might not be in favour of exported actin. To overcome this, we have tried 3' end replacement to tag endogenous actin. With this approach, we were also not able to detect the protein in the cytosol of the erythrocyte. We only manage to generate 3' end integrations with the small HA-tag and not with the big GFP-tag indicating that the GFP-tag most probably disrupts correct folding of actin or its polymerization leading to unfunctional actin. The 3' end HA-tag is possibly hidden within folded

actin and thus not properly accessible with the antibody in immunofluorescence assay and only detected in parasites where Actin-HA is over expressed as in parasites episomally expressing Actin-HA. The export of actin requires further elucidation but exported actin could play a role in transporting proteins across the host cell cytosol potentially being involved in vesicle formation and transport. Studies in yeast and higher eukaryotes have shown that actin functions at distinct and sequential steps in clathrin-mediated endocytosis. Specifically, actin polymerization drives membrane invagination, possibly scission, and then post-scission movement of the newly formed vesicle (for review see (Robertson *et al.*, 2009)).

In addition to actin we also pulled down the Ring Infected Erythrocyte Surface Antigen (RESA), a putative RESA, and High Molecular Weight Rhoptry Protein 2 (RhopH2). RhopH2 has a predicted molecular weight of 163 kD and has been described to localize to rhoptries (Ling *et al.*, 2003). RESA has no orthologs in other *Plasmodium* species and is synthesized in mature parasites where it is stored in dense granules (Culvenor *et al.*, 1991). Upon invasion RESA is released into the host cell cytosol where it associates with the erythrocyte membrane interacting with the spectrin network (Foley *et al.*, 1991). It plays a major role in reducing deformability of the erythrocyte at ring stage (Mills *et al.*, 2007). We have not yet verified whether these proteins truly interact with MAHRP2 although an interaction of RESA with MAHRP2 would be plausible. As MAHRP2 localizes to structures that seem to connect Maurer's clefts to the erythrocyte membrane it would only make sense to have interacting partners at Maurer's clefts (potentially PF07_0008) and at the host cell membrane (hypothetically RESA) anchoring the tubular structure.

However, more research is needed to shed more light on interacting partners of MAHRP2. Ongoing studies include co-immunoprecipitation studies to get more of a real life situation.

MAHRP2 knock down parasites

The importance of MAHRP2 was highlighted by the fact that all attempts to conventionally knock out the gene failed. Therefore, we wanted to establish a

conditional knock out system to study the function of the protein in this way. We have made use of the recently established FKBP degradation domain system (Armstrong & Goldberg, 2007, Banaszynski *et al*., 2006, Dvorin *et al*., 2010, Herm-Gotz *et al*., 2007, Russo *et al*., 2009). Proteins fused to the destabilizing domain (DD) FKBP are rapidly degraded in the absence of the ligand Shield-1 (Shld) whilst they should be stabilized in its presence. This system has been shown to work efficiently for regulating protein levels of soluble or membrane associated but not exported proteins in *P. falciparum* (Armstrong & Goldberg, 2007, Dvorin *et al*., 2010, Russo *et al*., 2009) and *Toxoplasma gondii* (Herm-Gotz *et al*., 2007). We were able to regulate the level of cytosolic YFP-DD by the addition or removal of Shld. In contrast, we were not able to regulate MAHRP2 protein levels. MAHRP2-DD fusion proteins were not stabilized upon addition of Shld resulting in an enormous decrease of MAHRP2 protein to minute levels. MAHRP2 protein was almost absent resulting coincidentally in an 'almost' knock out parasite. We observed no difference in *mahrp2* RNA levels as compared to wild type parasites demonstrating that the degradation system was working (with or without Shld). Minute residual amounts of protein were present at all times and could be detected using an antibody against the C-terminal Myc tag. Using the less sensitive antibody against MAHRP2 did not yield a signal in Western blots even when large amounts of parasite lysate were loaded. Shld seems to be unable to access the destabilizing domain of MAHRP2-DD, probably because it might be hidden on its transit through the secretory pathway. This supports the hypothesis that the protein is co-translationally translocated into the ER lumen and so far, no Shld-dependent regulation of exported proteins in *Plasmodium* has been demonstrated.

An almost complete MAHRP2 knock down parasite raises the question about the essentiality of MAHRP2. Obviously, residual protein remains and this seems to be sufficient to fulfil the function of the protein provided it is essential. Our preliminary analyses did not reveal any obvious altered phenotype for the knock down parasites. Other exported proteins were found to be normally exported and initial growth rate experiments indicated that growth is comparable to wild type parasites.

Why should a protein like MAHRP2 be essential for parasite survival in culture? Deletion of genes encoding Maurer's cleft resident proteins also did not affect parasite survival *in vitro* (Cooke *et al.*, 2006, Maier *et al.*, 2009, Maier *et al.*, 2007, Spycher *et al.*, 2008). Maurer's clefts are thought to play a crucial role in the transport of parasite derived proteins to the surface of the erythrocyte. A similar role could also be proposed for tethers in that proteins might be trafficked from Maurer's clefts along tethers to the surface. Deletion mutants of Maurer's clefts proteins never lost the formation of the clefts although structural differences were observed. Electron microscopy will shed light on whether the formation of tethers in the MAHRP2 knock down parasites is impaired. However, one could speculate that tethers may not be involved in protein export, but in the essential nutrient import. *Plasmodium* cannot solely survive by digestion of haemoglobin, it also needs to import nutrients from the extra cellular matrix (Desai *et al.*, 2000, Kirk & Saliba, 2007, Martin & Kirk, 2007). Very little is known about these mechanisms. It is thought that the change in permeability of the erythrocyte membrane induced by the formation of new permeability pathways is a process required for nutrient uptake (Kirk & Saliba, 2007, Martin & Kirk, 2007). Interestingly, preliminary observations indicated that erythrocytes infected with MAHRP2 knock down parasites were not as susceptible to sorbitol lysis as erythrocytes infected with wild type parasites. Sorbitol lysis is based on the permeability change of the host cell membrane induced by the parasite. Whether tethers or MAHRP2 play a role in the formation of new permeability pathways remains yet elusive. If fewer tethers are present in the knock down parasites this could explain the preliminary observation of altered sorbitol susceptibility. This hypothesis needs to be pursued and more experiments are needed.

In conclusion, we demonstrate that the hydrophobic domain of MAHRP2 is a functional transmembrane domain in the context of the integral membrane protein MAHRP1. MAHRP2 is trafficked through the ER on its route to tethers in the erythrocyte cytosol. Proteins potentially interacting with MAHRP2 have been found, among those being the two PNEPs PF07_0008 and phistc PF08_0137. *Pf*Actin is another possible partner which might be exported to Maurer's clefts

but this awaits further confirmation. Furthermore, we were able to knock down MAHRP2 protein levels using a conditional knock out system. Unravelling the trafficking mechanisms and the function of exported proteins involved in host cell refurbishment will lead to a better understanding of the early events after invasion and advancement in finding novel drug targets for malaria intervention.

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Figure Legends

Figure 1. Replacing the transmembrane domain of MAHRP1 with the hydrophobic domain of MAHRP2 does not change its location. For the MAHRP1(M2TM)GFP (A) transfectants dual labelling was performed with anti-GFP antibodies (green), anti-MAHRP2 antibodies (red, top row), and anti-MAHRP1 antibodies (red, bottom row). Scale bar = 5 µm. (B) Western blots of Triton X-114-fractionated protein extracts from 3D7 and MAHRP1(M2TM)GFP expressing parasites were probed with mouse anti-GFP antibodies (upper panel) and rabbit anti-MAHRP1 antibodies (lower panel). MAHRP1(M2TM)GFP was partly recovered in the detergent phase.

Figure 2. MAHRP2 export is brefeldin A sensitive. Immunofluorescence microscopy of acetone/methanol fixed smears after brefeldin A (BFA) treatment of erythrocytes infected with MAHRP2-GFP transfectants. Parasites were labelled with mouse anti-GFP antibodies (green) and DAPI (blue). Scale bar = 5 µm.

Figure 3. MAHRP2 is exported through the ER. (A, D) Western blot analysis of saponin-insoluble material from erythrocytes infected with SP-M2-GFP and SP-M2ΔTM-GFP transfectants (A) or SP-M1-GFP and SP-M1ΔTM-GFP transfectants (D) probed with mouse anti-GFP (upper panels) and mouse anti-GAPDH (lower panels) antibodies. (B, C, E) Immunofluorescence microscopy of acetone/methanol fixed smears of erythrocytes infected with SP-M2-GFP (B), SP-M2ΔTM-GFP (C) or SP-M1-GFP (E, upper panel) and SP-M1ΔTM-GFP (E, lower panel) transfectants. Parasites were labelled with mouse anti-GFP (green), rabbit anti-MAHRP1 (red, A lower panel) antibodies, and DAPI (blue). Scale bar = 5 µm.

Figure 4. Mutation of the potential myristoylation site has no effect on export or solubility of MAHRP2. (A) Amphipathic wheel prediction of the 18 amino acids following glycine 16. (B) Western blots of Triton X-114-fractionated protein extracts from 3D7 wild type parasites and $M2_{G16A}$-GFP transfectants were probed with mouse anti-GFP, rabbit anti-MAHRP2 antibodies (left panel) and rabbit anti-MAHRP1 antibodies (right panel). A = aqueous phase, D = detergent phase. MAHRP2 as well as $M2_{G16A}$-GFP were found only in the aqueous phase. (C) Immunofluorescence microscopy of acetone/methanol fixed smears of

erythrocytes infected with $M2_{G16A}$-GFP transfectants. Parasites were labelled with mouse anti-GFP antibodies (green) and DAPI (blue). Scale bar = 5 µm.

Figure 5. Samples from pull down experiments were run on gradient acrylamide gels and stained with (A) SimplyBlue™ or (B) silver.

Figure 6. *Pf*Actin is potentially exported to Maurer's clefts as a candidate interaction partner of MAHRP2. (A) Western blot analysis of saponin-insoluble material from erythrocytes infected with 3D7 wild type and transfectants episomally expressing Actin-HA probed with rat anti-HA (left panel) and mouse anti-Actin (right panel) antibodies. (B) Dual label immunofluorescence microscopy of acetone/methanol-fixed 3D7 smears using antibodies recognizing MAHRP2 (green, panel 1), anti-REX1 (green, panel 2), and anti-Actin (red). Scale bar = 5 µm. (C) Immunofluorescence microscopy of acetone/methanol fixed smears of erythrocytes infected with Actin-HA (upper panel) and ActinIntHA (lower panel) transfectants. Parasites were labelled with rat anti-HA (red upper panel, green lower panel) or mouse anti-GFP (green) antibodies and DAPI (blue). Scale bar = 5 µm.

(D) Western blot analysis of saponin-insoluble material from erythrocytes infected with transfectants episomally expressing ActinIntHA (right panel) or endogenously tagged Actin3'HA (right panel) and Actin3'GFP (left panel) probed with rat anti-HA or mouse anti-GFP, and mouse anti-GAPDH antibodies.

Figure 7. Potential tether protein components were found by mass spectrometric analysis of the 80'000 *g* pellet from differential centrifugation experiment. (A) and (B) Western blot analysis of saponin-insoluble material from erythrocytes infected with transfectants (A) episomally expressing PF08_0137-GFP, MAL13P1_237-HA, and PF07_0008-HA probed with mouse anti-GFP (left panel) and rat anti-HA (right panel) antibodies; or (B) expressing a 3' end tagged PFL0930w-TY and 3D7 wild type parasites probed with mouse anti-TY and mouse anti-GAPDH antibodies. (C) Dual label immunofluorescence microscopy of acetone/methanol-fixed smears of erythrocytes infected with PF07_0008-HA (panel 1), PF08_0137-GFP (panels 2 and 3), and MAL13P1.237-HA (panel 4) transfectants using rat anti-HA (red, panels 1 and 4), mouse-anti GFP (green,

panels 2 and 3), rabbit anti-MAHRP1 (green, panel 1 and red, panel 3), and rabbit anti-MAHRP2 (red, panel 2 and green, panel 4) antibodies. Scale bar = 5 µm.

Figure 8. The *mahrp2* locus is efficiently targeted for integration with a *mahrp2-dd* fusion. Southern blot of EcoRI digested genomic DNA from 3D7 wild type and 3D7-M2DD parasites. (A) Digested genomic DNA was run on a 0.7% agarose gel and stained with ethidium bromide. (B) Southern blot demonstrating a successful 3' end replacement of the endogenous *mahrp2* locus. The blot was probed with biotinylated *mahrp2* and *hdhfr* probes. (C) Genomic maps of the *mahrp2* locus of 3D7 wild type (upper panel) and 3D7-M2DD (lower panel) parasites.

Figure 9. MAHRP2 protein is almost completely absent in M2DD transfectants. (A) Western blot analysis of saponin-insoluble material from erythrocytes infected with M2DD transfectants and wild type parasites cultured in the presence and absence of Shield-1 were probed with mouse anti-GAPDH (left panel), mouse anti-Myc (middle panel), and rabbit anti-MAHRP2 (right panel) antibodies. (B and C) Immunofluorescence microscopy of acetone/methanol fixed smears of erythrocytes infected with M2DD transfectants (B) and wild type parasites (C). Parasites were labelled with rabbit anti-MAHRP2 antibodies (green) and DAPI (blue). Scale bar = 5 µm. (D) Western blot analysis of saponin-insoluble material from erythrocytes infected with YFP-DD transfectants cultured in the presence and absence of Shield-1 were probed with mouse anti-GFP and mouse anti-GAPDH antibodies.

Figure 10. The export of other proteins is found not to be affected by the absence of MAHRP2. Immunofluorescence microscopy of acetone/methanol fixed smears of erythrocytes infected with M2DD transfectants (upper row in panels) and wild type parasites (lower row in panels). Parasites were labelled with mouse anti-*Pf*EMP1 (panel 1), mouse anti-KAHRP (panel 2), rabbit anti-MAHRP1 (panel 3), and mouse-anti SBP1 (panel 4) antibodies (red). Scale bar = 5

Supplementary Figure 1. Symbolic representation of the different transfection constructs. MAHRP2: N-terminal domain is shown in yellow, the C-terminal domain in blue, and the hydrophobic stretch in red. MAHRP1: N-terminal domain is shown in light blue, the C-terminal domain in light yellow and the transmembrane

domain in purple. The MSP1 signal peptide is indicated in grey, the DD-FKBP domain in orange, the Myc- and TY-tag in light pink, the HA-tag in pink, and GFP in green.

Supplementary Table 1. Primers and restriction sites used to generate the various constructs are listed in this Table. The steps that were done for PCR amplification is indicated in the last column.

Supplementary Table 2. Mass spectrometry results of pull down with recombinant MAHRP2. Peptide hits of potential protein interaction partners are listed with their quality score (good quality, Xc > 2 and ΔCn > 0.1).

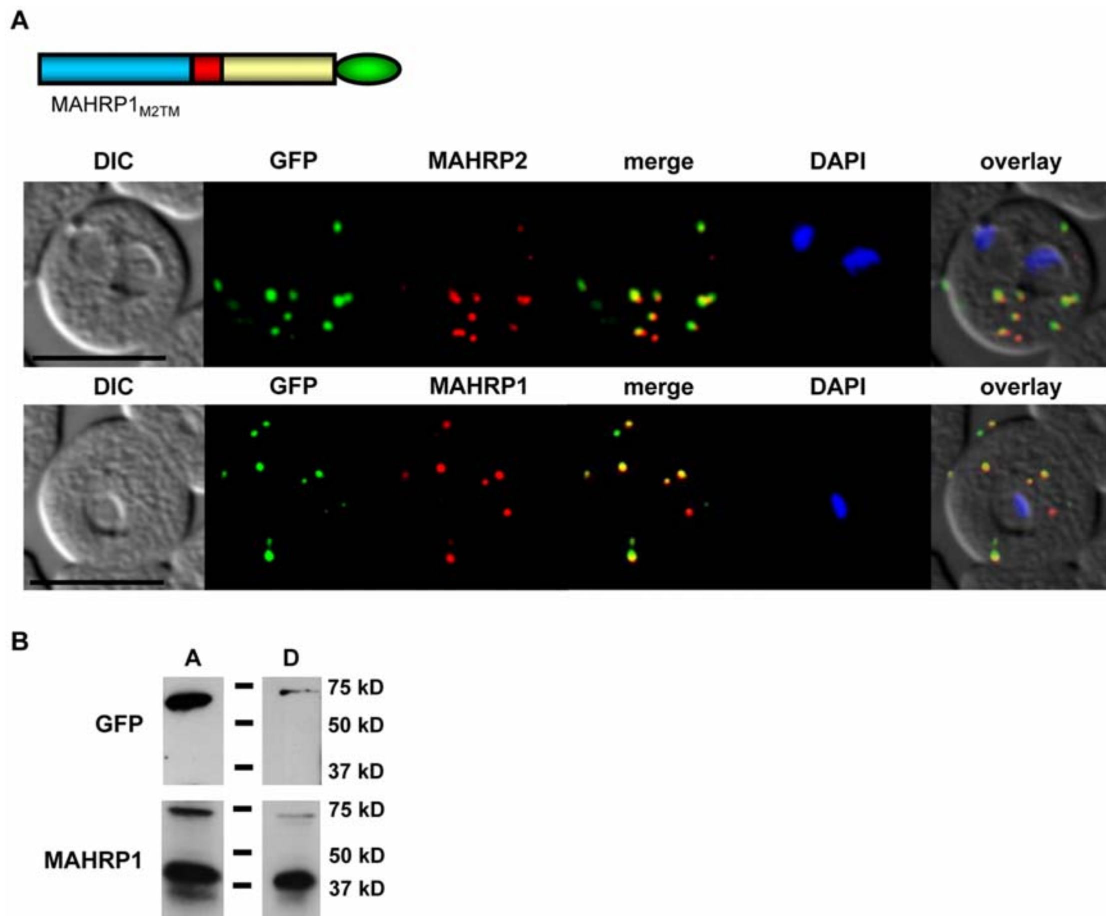


Figure 1

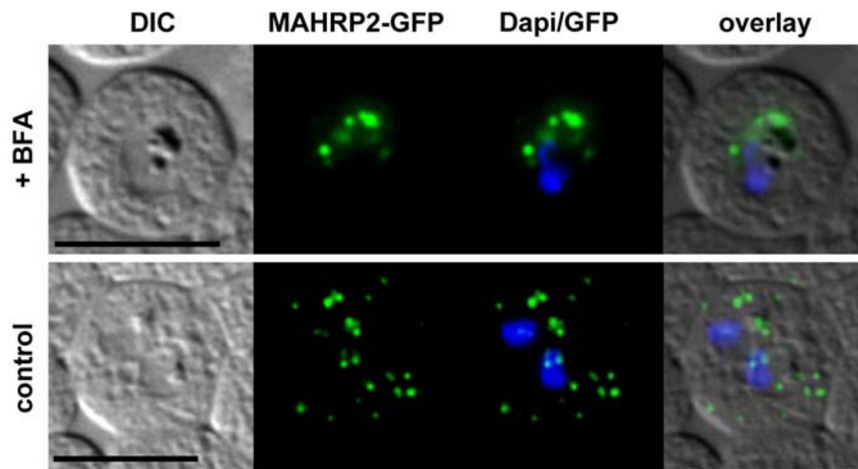
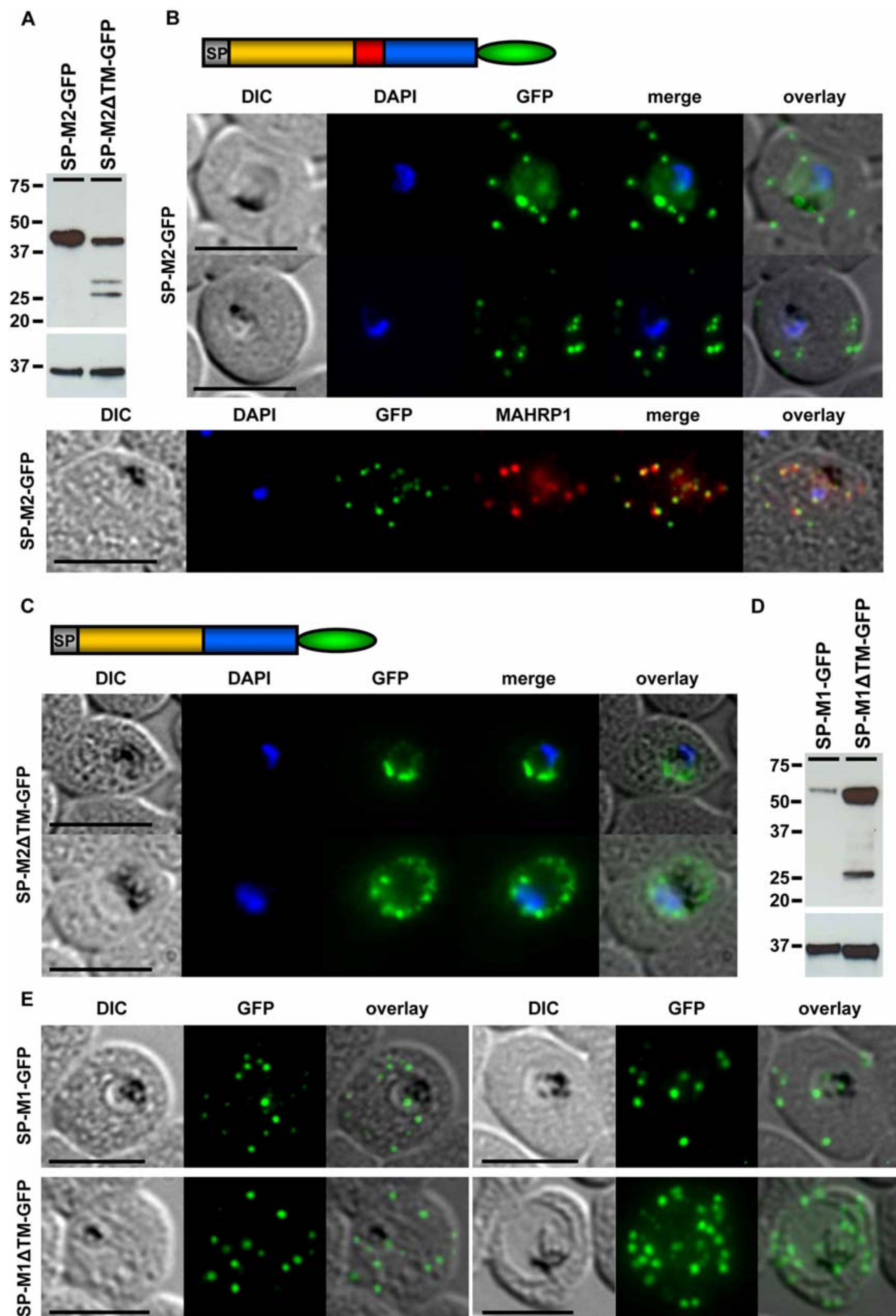


Figure 2



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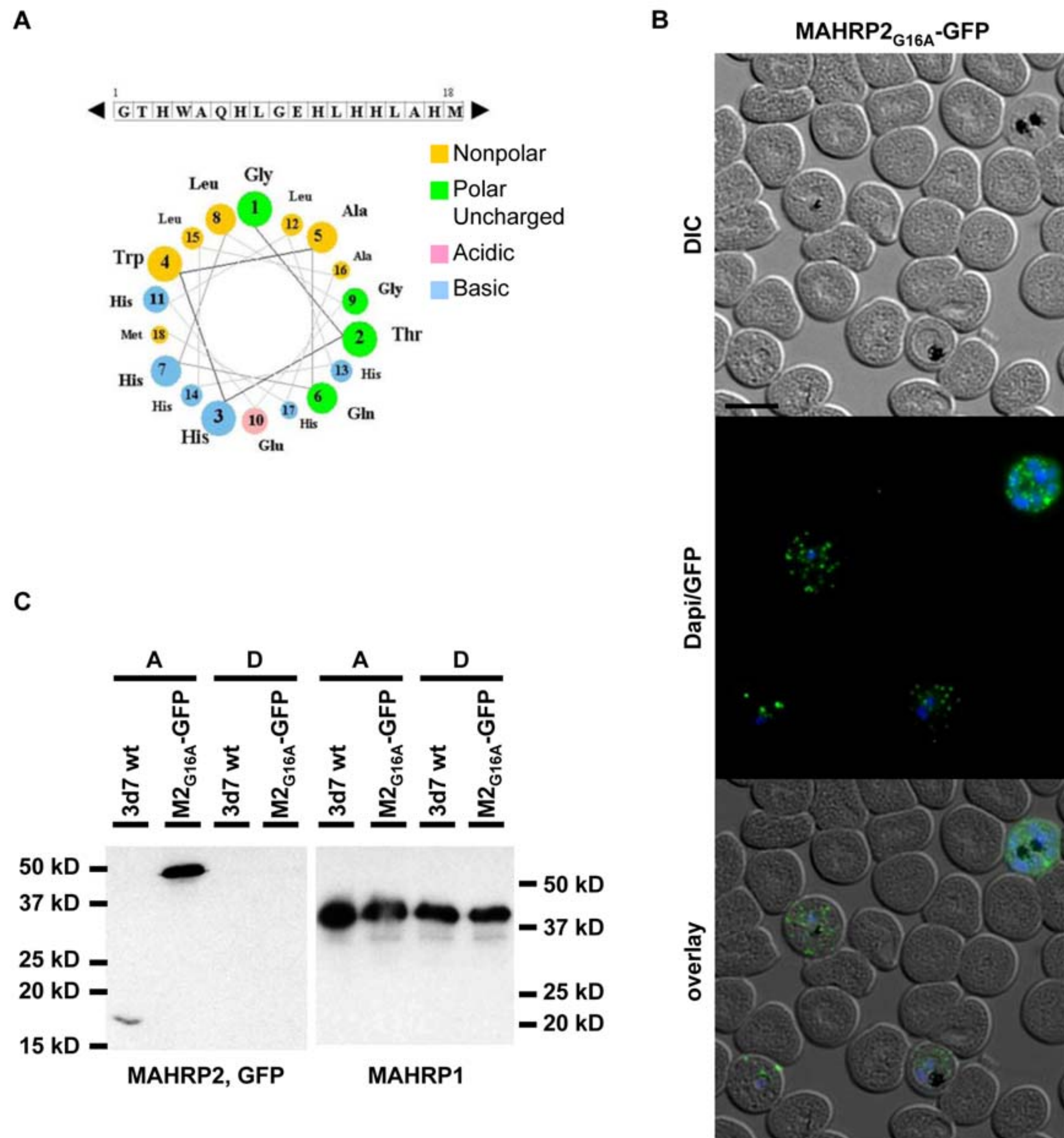

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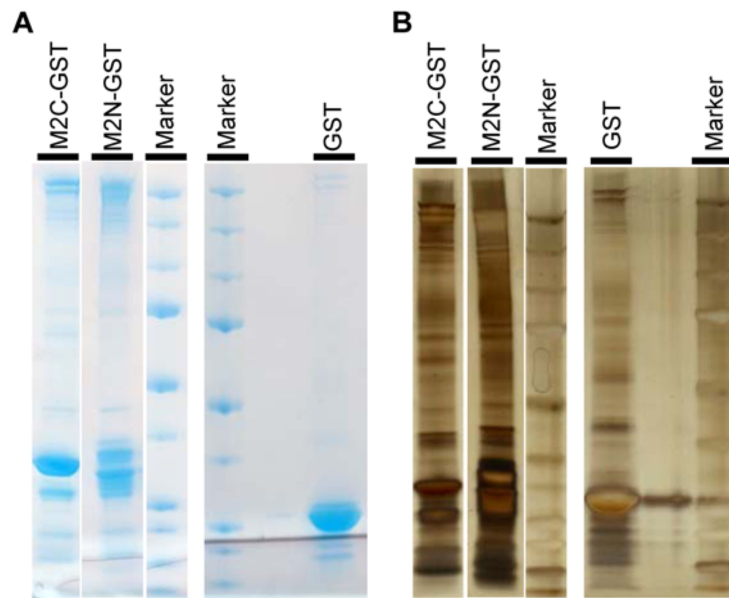

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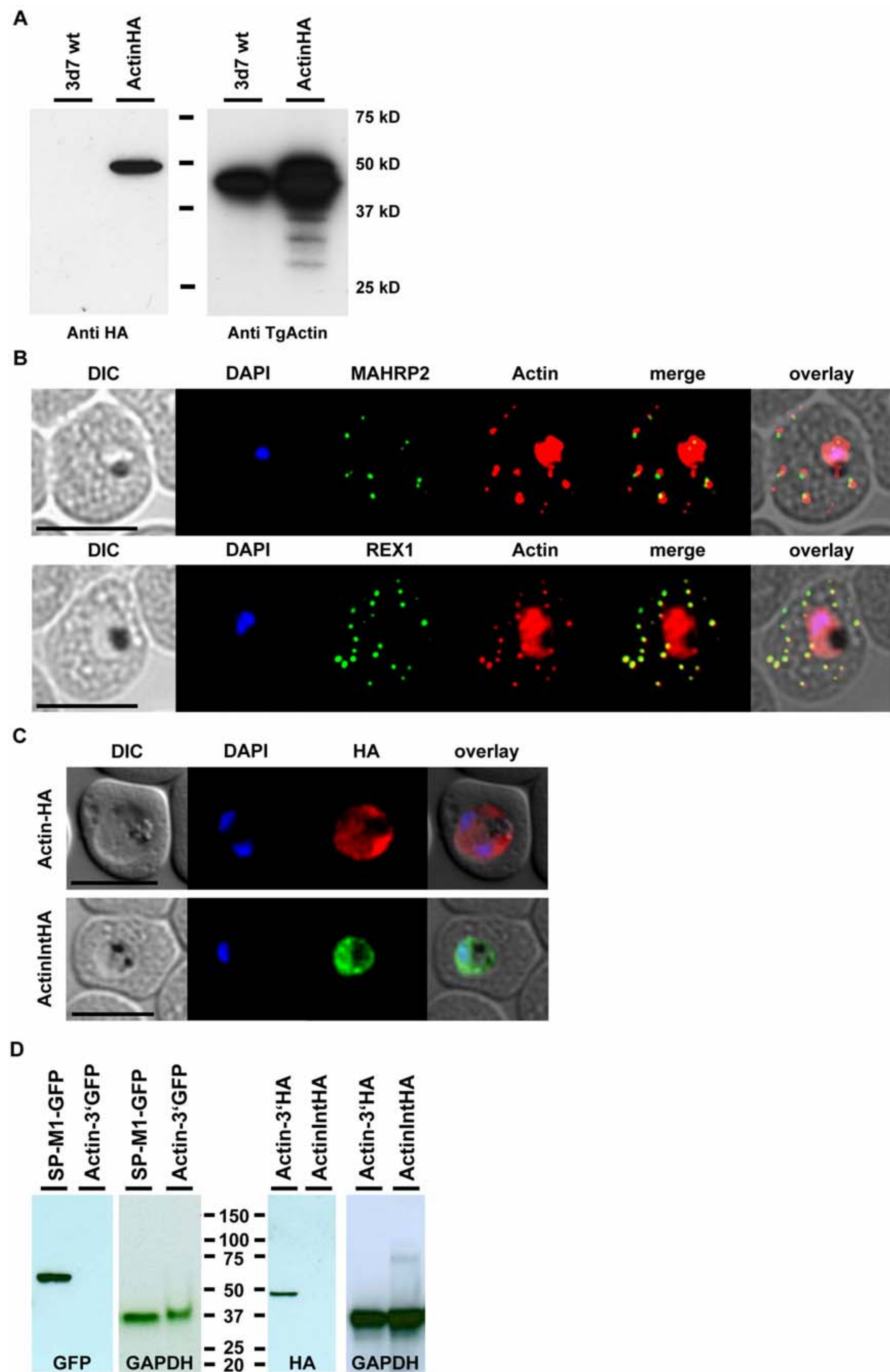

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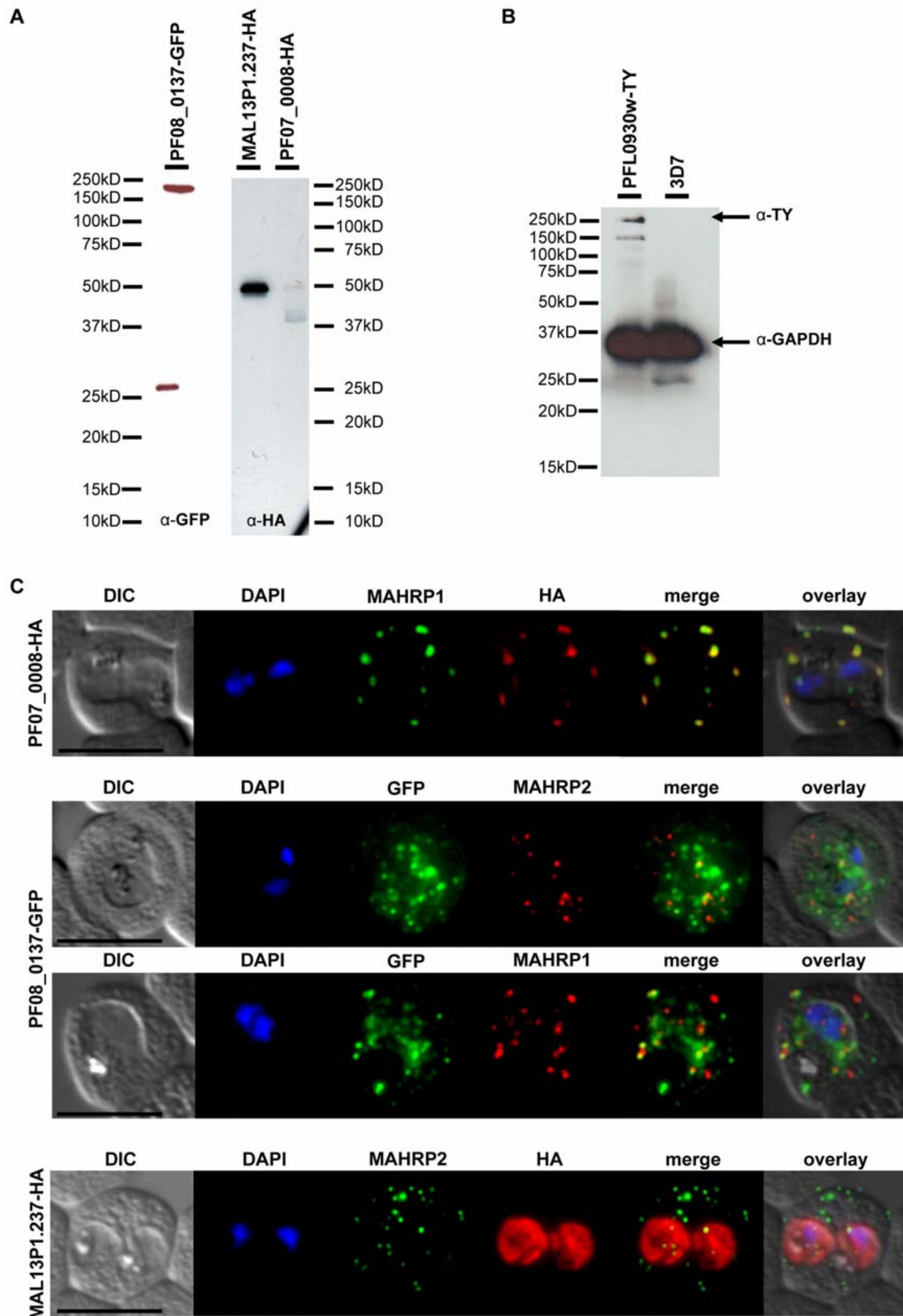


Figure 7

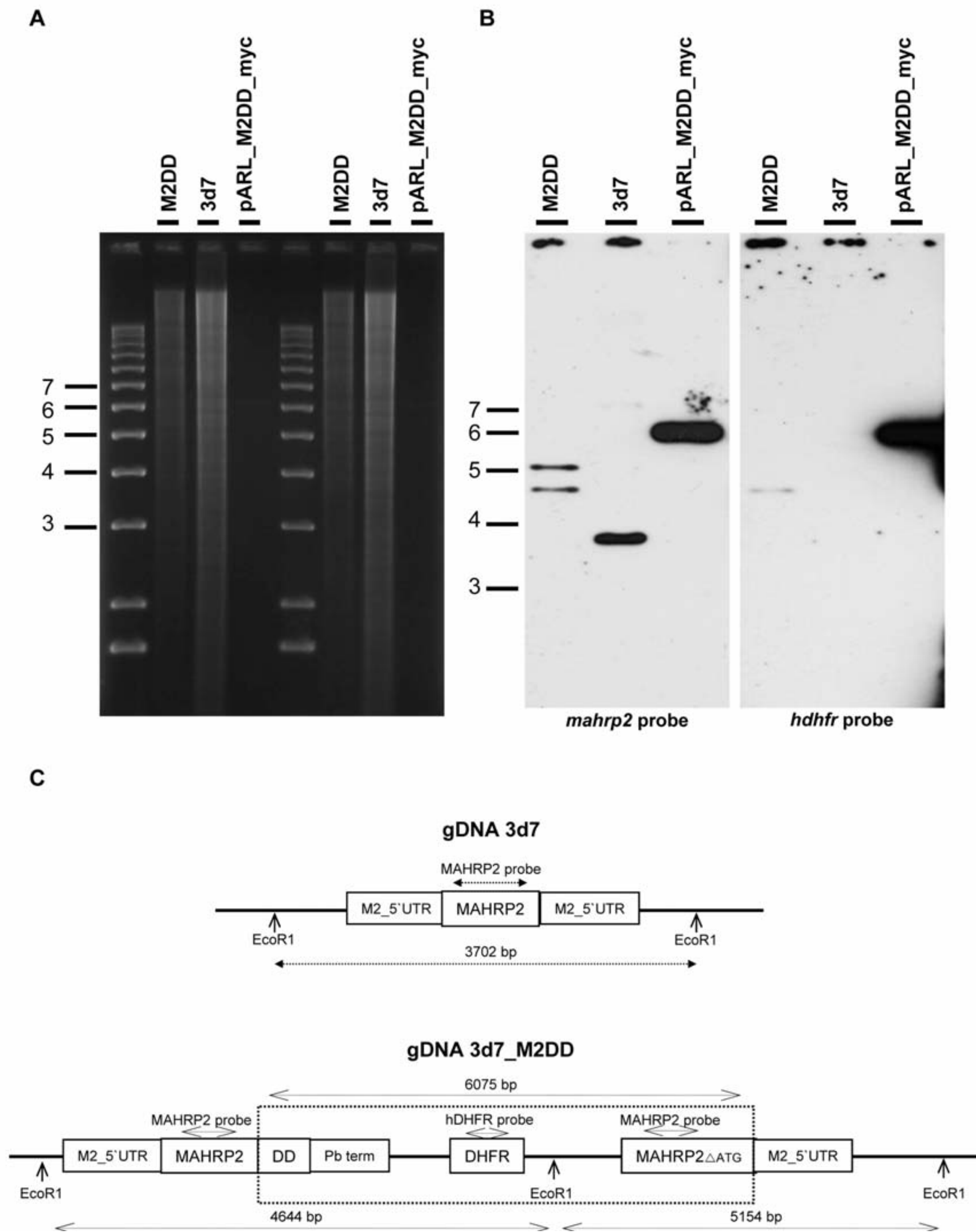


Figure 8

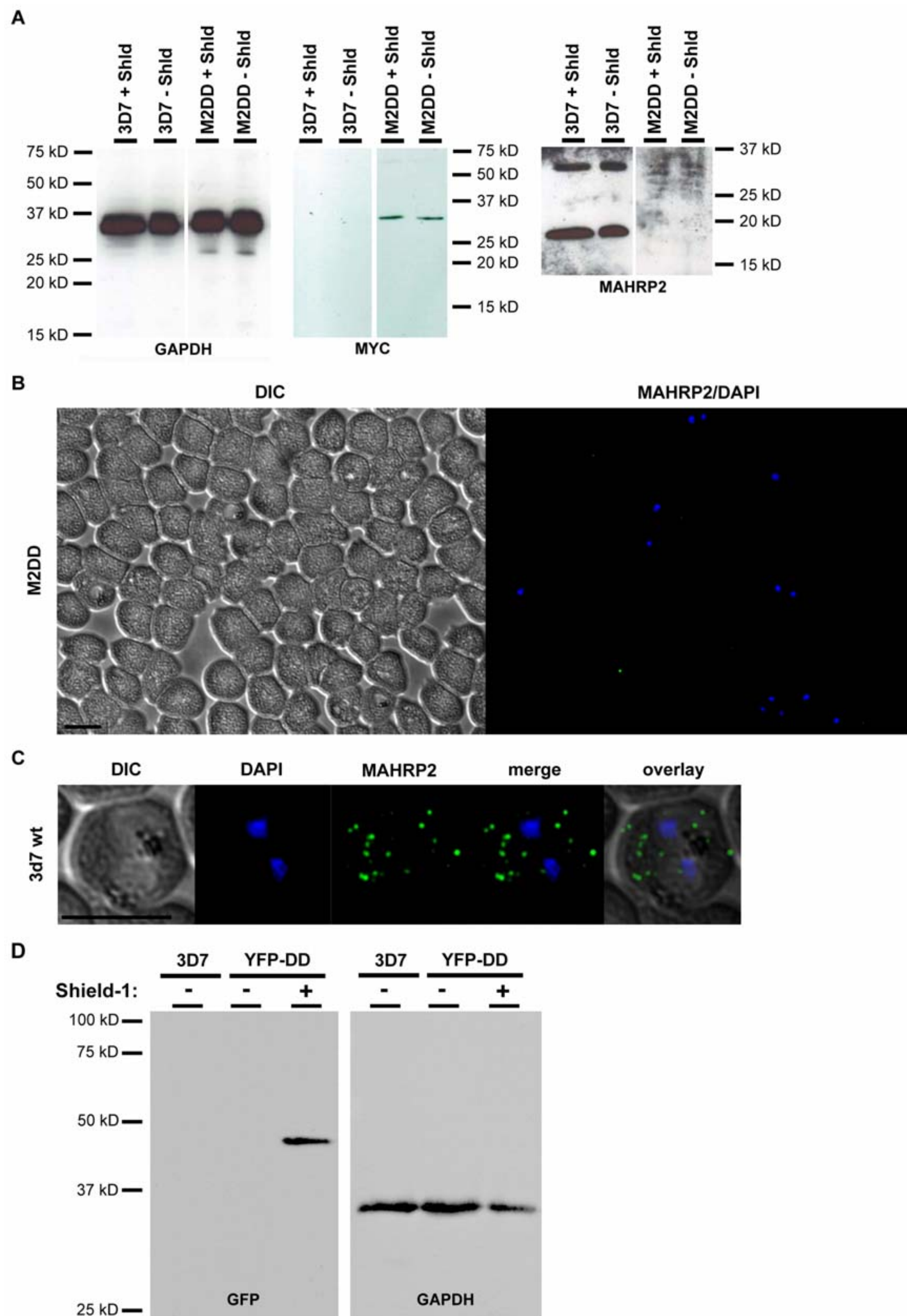


Figure 9

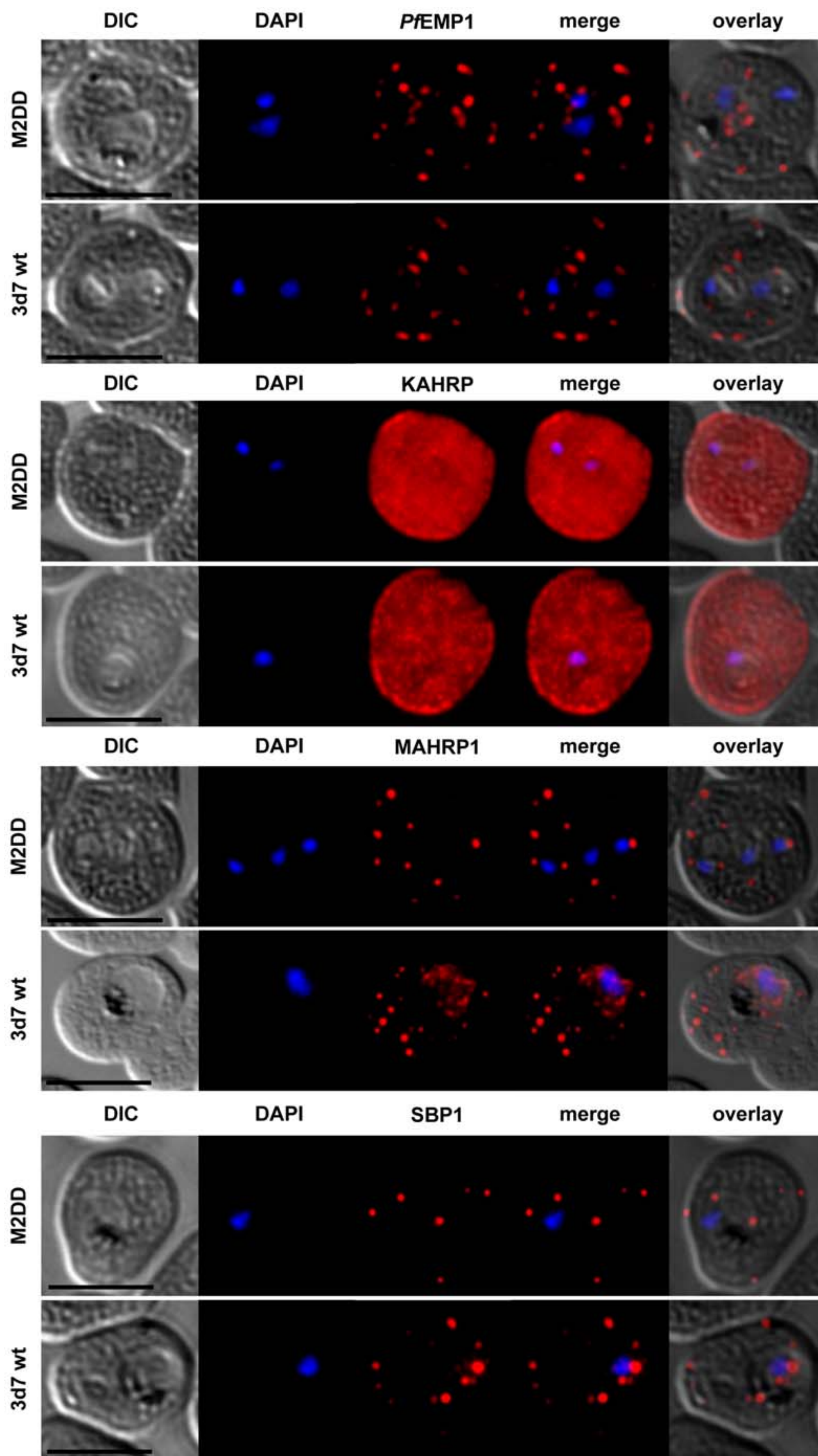
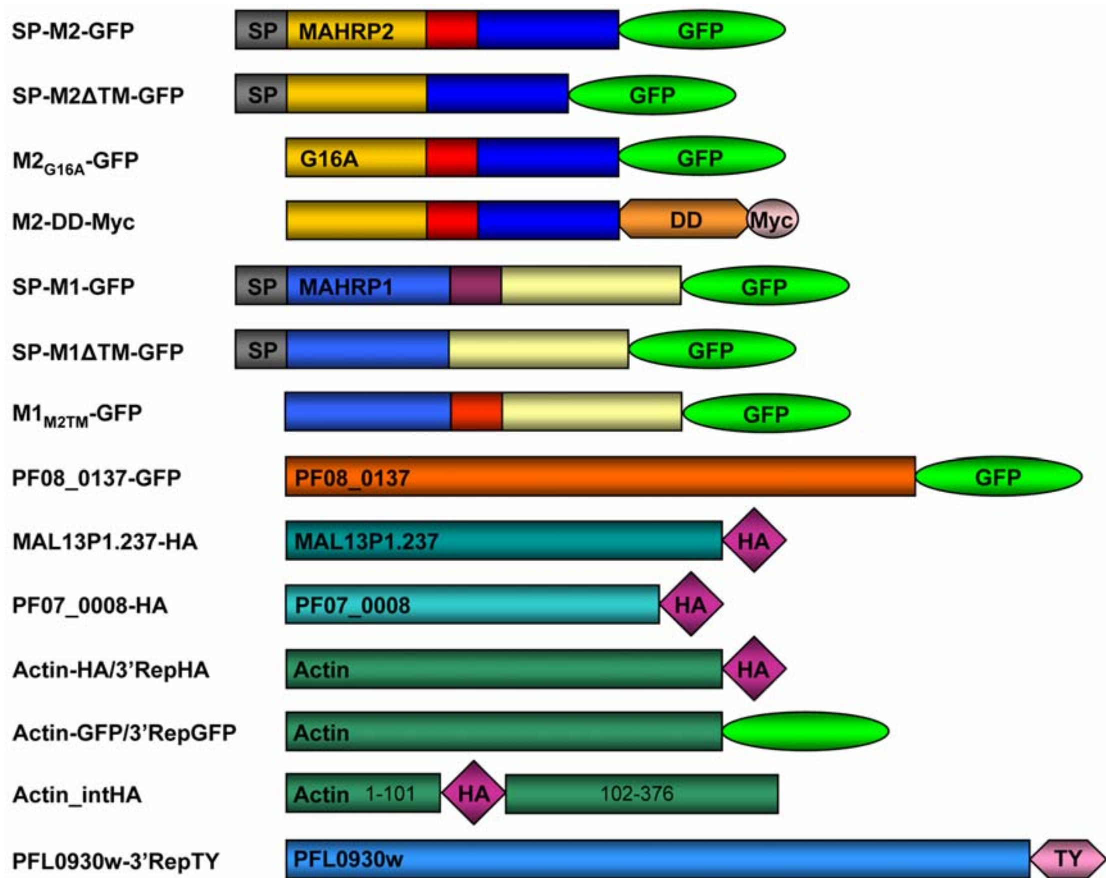


Figure 10



Supplementary Figure 1

Gene	Construct	Primer name	Primer sequences	Restr. sites	Comments
$M1_{M2TM}$	pARL_$M1_{M2TM}$_GFP	53f	atatcttaagATGGCAGAGCAAGCAGC	AflII	PCR1 (53f + 55r) and PCR2 (56f + 54r) were done separately under normal conditions. The two PCR products were then used mixed together as template for a 3rd PCR using primers 53f + 54r
		55r	ATATAATCTATAAACTACTACTAGGAAGAAAGCCAATAATAAAAAGATTAACATTAAATGATTCTTTCCATCTAAAGCTTCAG		
		56r	CATTTAATGTTAATCTTTTTATTATTGGCTTTCTTCCTAGTAGTAGTTTATAGATTATATGCCTTCTTATACCACTCAgtaag		
		54r new	atatatcgatATTATCTTTTTTTTCTTGTTC	ClaI	
$M2_{G16A}$	pARL_$M2_{G16A}$_GFP	40f	GCAACTCATTGGGCTCAACATTTAG		PCR1 was done using primers 40f and 34r. The product was used as template for the final PCR reaction using primers 41f and 34r.
		41f	ATCTTAAGATGCAGCCTTGTCCATATGATGTATACAATCAAATAAACCATGTAGCAACTCATTGGGCTCAAC	AflII	
		34r	ATATATCGATtaatgtttgttgtgtactagtag	ClaI	
SP-M2	pARL_SP-M2_GFP	70f	atacttaagATGAAGATCATATTCTTTTTATGTTCATTTCTTTTTTTTATTATAAATACACAATGTGTACAGCCTTGTCCATATGATG	AflII	Regular PCR reaction
		34r	ATATATCGATtaatgtttgttgtgtactagtag	ClaI	
SP-M2ΔTM	pARL_SP-M2ΔTM_GFP	70f	atacttaagATGAAGATCATATTCTTTTTATGTTCATTTCTTTTTTTTATTATAAATACACAATGTGTACAGCCTTGTCCATATGATG	AflII	PCR1 (70f + 71r) and PCR2 (72f + 34r) were done separately under normal conditions. The two PCR products were then used mixed together as template for a 3rd PCR using primers 70f + 34r
		71r	cttatatatgttaataataacttacCTCGTTGGAACTACAATGTTCATGATG		
		72f	GTCGACATCATGAACATTGTAGTTCCAACGAGgtaagttattattaac		
		34r	ATATATCGATtaatgtttgttgtgtactagtag	ClaI	
SP-M1	pARL_SP-M1_GFP	73f	atacttaagATGAAGATCATATTCTTTTTATGTTCATTTCTTTTTTTTATTATAAATACACAATGTGTAGCAGAGCAAGCAGCAGTAC	AflII	Regular PCR reaction
		54r new	atatatcgatATTATCTTTTTTTTCTTGTTC	ClaI	
SP-M1ΔTM	pARL_SP-M1ΔTM_GFP	73f	atacttaagATGAAGATCATATTCTTTTTATGTTCATTTCTTTTTTTTATTATAAATACACAATGTGTAGCAGAGCAAGCAGCAGTAC	AflII	PCR1 (73f + 74r) and PCR2 (75f + 54r) were done separately under normal conditions. The two PCR products were then used mixed together as template for a 3rd PCR using primers 73f + 54r
		74r	taatgaacttacTGAGTGGTATAAGAAGGCATTCTTTCCATCTAAAGCTTCAG		
		75f	CTCTTTCTGAAGCTTTAGATGGAAAGAATGCCTTCTTATACCACTCAgtaag		
		54r	atatatcgatATTATCTTTTTTTTCTTGTTC	ClaI	
PF08_0137	pARL_PF08_0137_GFP	44f	ATATCTTAAGATGATATTTGTTAAGAGTAAGATTTTATATTTC	AflII	Regular PCR reaction
		45r	ATATATCGATTGCTCCACTATATTTTCTCATTTTATAAG	ClaI	
MAL13P1_237	pBcam_MAL13P1_237_HA	46f	ATATGGATCCATGGAAAATGATAAAAAACATAAC	BamHI	Regular PCR reaction
		47r	ATATCCATGGTTGCTCATTGTGATTGAAAAC	NcoI	
PF07_0008	pBcam_PF07_0008_HA	48f	ATATGGATCCATGGCTTATCCTCTTTTAGAAGATG	BamHI	Regular PCR reaction
		49r	ATATGCGGCCGCTACATGAGCTTCATTAGTGTTTAAAC	NotI	
actin	pBcam_Actin_HA	42f	ATATGGATCCATGGGAGAAGAAGATGTTCAAG	BamHI	Regular PCR reaction
		43r	ATATCCATGGGAAACATTTTCTGTGGACAATAC	NcoI	
3' replacement of *actin*	pARL_Actin3'GFP	60f	actGCGGCCGCttgctattcaagctgttttatc	NotI	Regular PCR reaction
		62r	atatcttaaggaaacattttctgtggacaatac	AflII	
3' HA replacement of *actin*	pARL_Actin3'HA	60f	actGCGGCCGCttgctattcaagctgttttatc	NotI	Regular PCR reaction
		61r	atatactagtttagagctcggcataatctg	SpeI	
actin_intHA	pBcam_ActinIntHA	42f	ATATGGATCCATGGGAGAAGAAGATGTTCAAG	BamHI	PCR1 (42f +66r) and PCR2 (67f + 65r) were done separately under normal conditions. The two PCR products were then used mixed together as template for a 3rd PCR using primers 42f + 65r
		66r	cgcataatcgggcacatcatagggatagccagcgtagtccgggacgtcgtacgggtaTTCTTCTGGAGCAGCTCTTAATTC		
		67f	tacccgtacgacgtcccggactacgctggctatccctatgatgtgcccgattatgcgCACCCAGTGTTATTAACAGAAG		
		65r	atatgagctcTTAGAAACATTTTCTGTGGAC	SacI	
mahrp2	pARL_mahrp2_FKBP_myc	MAHRP2-f-NO_ATG	AAAAGATCTCAGCCTTGTCCATATGATG	BglII	Regular PCR reaction
		MAHRP2-r-NO_TAA	AAACCTAGGTAATGTTTGTTGTGTACTAGTAG	AvrII	

Supplementary Table 1

MAHRP2 N-terminal domain

Protein	Peptide	Score XC	Coverage DeltaCn	MW Sp	Peptide (Hits) Ions
High molecular weight rhoptry protein-2	PFI1445w | organism=Plasmodium falciparum_3D7	50.20			5 (5 0 0 0 0)
	K.SLYGNNNNNNAGSESDVTLK.S	3.99	0.73	1199.1	21/38
	K.DCNVNQNFTETSK.K	3.48	0.46	1606.1	20/24
	K.TQYEENKLSEYR.A	3.14	0.60	1065.7	16/22
	K.NIVSDALTSEEIKR.H	2.72	0.59	592.0	14/26
	K.GSQNTEGESEVPSDDEINK.T	2.54	0.47	297.1	16/36
ring-infected erythrocyte surface antigen	PFA0110w | organism=Plasmodium falciparum_3D7	50.20			5 (5 0 0 0 0)
	K.VNEAYQVLGDIDK.K	4.07	0.62	1789.6	19/24
	K.NISDLSYTDQKEILEK.I	4.00	0.59	1675.7	20/30
	K.ALSNQIQYSCR.K	2.96	0.62	672.5	14/20
	K.LIDQGGENLEER.L	2.46	0.37	247.3	12/22
	K.LAENYYPYQR.S	2.41	0.57	399.3	12/18
actin	PFL2215w | organism=Plasmodium falciparum_3D7	20.13			2 (2 0 0 0 0)
	R.AAPEEHPVLLTEAPLNPK.G	2.67	0.56	707.2	18/34
	K.IWHHTFYNELR.A	2.16	0.54	390.6	11/20

MAHRP2 C-terminal domain

Protein	Peptide	Score XC	Coverage DeltaCn	MW Sp	Peptide (Hits) Ions
High molecular weight rhoptry protein-2	psu|PFI1445w | organism=Plasmodium falciparum_3D7	100.24			10 (10 0 0 0 0)
	K.SLYGNNNNNNAGSESDVTLK.S	4.57	0.71	1648.6	24/38
	K.NNDQLTFLETQVAK.I	3.95	0.69	1202.0	18/26
	K.NIVSDALTSEEIKR.H	3.48	0.73	1609.7	19/26
	K.DFTQTNALTNLPNLDNK.K	3.32	0.48	883.4	18/32
	K.DCNVNQNFTETSK.K	3.30	0.42	1100.7	19/24
	K.GSQNTEGESEVPSDDEINK.T	3.17	0.45	467.2	18/36
	K.LFEQIVDQIK.Y	2.87	0.29	867.0	14/18
	R.SNNIVALYILK.T	2.84	0.49	860.1	15/20
	K.TQYEENKLSEYR.A	2.22	0.40	569.5	12/22
	K.QLDDEEIER.F	2.07	0.21	214.7	10/16
ring-infected erythrocyte surface antigen	psu|PFA0110w | organism=Plasmodium falciparum_3D7	40.21			4 (4 0 0 0 0)
	K.NISDLSYTDQKEILEK.I	4.15	0.53	1786.3	21/30
	K.ALSNQIQYSCR.K	2.84	0.68	1000.6	14/20
	K.VNEAYQVLGDIDK.K	2.55	0.54	522.4	13/24
	K.LAENYYPYQR.S	2.20	0.61	531.9	12/18
putative ring-infected erythrocyte surface antigen	psu|PF11_0509 | organism=Plasmodium falciparum_3D7	30.17			3 (3 0 0 0 0)
	K.NSAESILTVEGLDEK.K	3.46	0.45	929.5	18/28
	K.ALSNQIQYTCR.K	2.41	0.51	468.4	13/20
	R.ETYISENLISR.L	2.34	0.26	316.7	10/20
MAHRP2	psu|PF13_0276 | organism=Plasmodium falciparum_3D7	50.28			5 (5 0 0 0 0)
	R.IVNITPVNEEHKAEASKEQSK.S	5.65	0.73	1764.8	25/40
	R.IVNITPVNEEHKAEASK.E	4.41	0.68	1053.5	23/32
	R.IVNITPVNEEHK.A	3.14	0.42	1088.7	18/22
	K.STSDSSTSTQQTL.-	3.03	0.61	389.4	16/24
	K.AEASKEQSK.S	2.19	0.46	570.5	12/16

Supplementary Table 2

Chapter 5

General Discussion

General Discussion

Today, malaria is still one of the major causes of morbidity and death in the world. The disease is triggered by parasites of the *Plasmodium* species among which *P. falciparum* causes most severe forms of malaria. Responsible for the pathology are *P. falciparum* blood stages. During part of its life cycle the parasite resides in human erythrocytes where it replicates asexually. The terminally differentiated erythrocyte is in a way simply a 'bag' filled with haemoglobin lacking all organelles and a protein trafficking machinery. To survive in such an environment the parasite renovates its host cell by exporting numerous proteins, forming membranous structures in the erythrocyte cytosol called Maurer's clefts and knob structures on the surface of the erythrocyte. These knobs harbour the major virulence factor, *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1), which is exposed on the surface and mediates binding to endothelial receptors leading to adherence and sequestration of the infected cells. This causes clogging of blood capillaries accounting for severe forms of malaria leading to organ failure and cerebral malaria. The transport of this virulence factor is crucially dependent on Maurer's cleft which is an intermediate storage compartment for *Pf*EMP1. Maurer's clefts are believed to be linked to the erythrocyte cytoskeleton and recently novel tubular structures called tethers have been described in the cytosol of the infected erythrocyte that seem responsible for the attachment of Maurer's clefts to the erythrocyte membrane (Hanssen *et al.*, 2010, Hanssen *et al.*, 2008).

As renovation of the host cell is essential for parasite survival we are interested in the processes involved. Comprehension of these processes could open up a whole new field for malaria intervention strategies. The refurbishment is initiated right upon parasite invasion which inspired us to identify genes expressed at this time of the parasite life cycle as they could be directly involved in these processes. In a stage-specific cDNA library (Spielmann & Beck, 2000) 13 ring-stage specific genes were found including two hypothetical histidine-rich proteins which were termed Membrane Associated Histidine-Rich Proteins 1 and 2 (MAHRP1 and MAHRP2).

The aim of this thesis was to characterize both proteins. Previous studies had shown that MAHRP1 is a Maurer's clefts resident protein (Spycher *et al.*, 2003). In this thesis, we further characterized possible roles of MAHRP1 in host cell refurbishment and studied the location of MAHRP2 in the infected erythrocyte. Not much is known about how proteins are secreted across the complex membrane system that includes the plasma membrane, the parasitophorous vacuole (PV), the PV membrane (PVM), Maurer's clefts, and the erythrocyte membrane. Interestingly, MAHRP1 and MAHRP2 both lack the recently described PEXEL (Marti *et al.*, 2004) or VTS (Hiller *et al.*, 2004) motif that promotes translocation across the PVM in most exported proteins of *P. falciparum* as well as a classical signal sequence. We therefore investigated how these PEXEL-negative proteins (PNEP) are trafficked to their destination (Pachlatko *et al.*, 2010, Spycher *et al.*, 2006).

Major findings

We generated parasite lines in which the *mahrp1* gene was disrupted to investigate possible functions of MAHRP1 in these knock out parasites. We found that in erythrocytes infected with MAHRP1 deficient parasites Maurer's clefts looked swollen and fragmented. Importantly, the major virulence factor *Pf*EMP1 was not exported to the surface of the erythrocyte anymore. It was still produced but was trapped within the confines of the parasite. This resulted in a reduced ability of the infected erythrocytes to bind to the endothelial receptor CD36. Complementation of the MAHRP1 deficient parasites with episomal expression of the gene could restore this phenotype.

Using antibodies raised against MAHRP2 as well as through transfection technology we could show that the protein was exported to recently described tethers. Importantly, MAHRP2 is the first and only protein so far specifically localizing to these structures. Life cell imaging of infected erythrocytes expressing MAHRP2-GFP revealed both mobile and fixed populations of these structures that could be enriched by differential centrifugation. Solubilization studies showed that MAHRP2 is a peripherally associated membrane protein but does not span

membranes. The hydrophobic stretch was shown to be functional as a transmembrane domain in a different context. We failed to delete the *mahrp2* gene indicating an essential function for MAHRP2 in parasite survival strategies. By the implementation of a newly developed conditional knock out system MAHRP2 protein levels could by reduced significantly to minute residual amounts. Through pull down experiments and mass spectrometric analysis of the enriched tether fraction obtained by differential centrifugation, we found several potential protein interaction partners of MAHRP2.

The sequences required for export of MAHRP2 were determined using transfectants expressing truncated MAHRP2 fragments. The first 15 amino acids and the histidine-rich N-terminal region were necessary for correct trafficking together with a predicted central hydrophobic region. Membrane association seemed to be required for the export of MAHRP2. Moreover, trafficking of MAHRP1 and MAHRP2 was ER dependent.

The roles of MAHRP1 and MAHRP2 or Maurer's clefts and tethers in host cell refurbishment

In immunofluorescence assays and 3D reconstructions we found MAHRP1 and MAHRP2 signals in close proximity associating with each other. In trophozoite stage infected erythrocytes we rarely observed a cleft without a tether or a tether without a cleft. Most Maurer's clefts appeared to adjoin to a tether on each of the two edges. On the other hand, in electron microscopic 3D reconstruction studies Maurer's clefts were sometimes seen without tethers as well as some tethers appeared to have one or both ends free (Hanssen *et al.*, 2010). Thus, it was suggested that tethers are modular features that can exist independently of the Maurer's clefts and that these structural elements are generated as separate entities that can assemble to tether the Maurer's clefts to different membranes. Hanssen *et al.* described an interaction of the tubular tether structure with the erythrocyte membrane skeleton rather than a direct bilayer connection. Time lapse imaging revealed that Maurer's clefts and tethers are highly mobile in early trophozoite stages (Pachlatko *et al.*, 2010, Spycher *et al.*, 2006, Grüring *et al.*,

2010). It could be that Maurer's clefts detach from one site, possibly the PVM, and move to another location such as to the erythrocyte membrane as the parasite matures. Maurer's clefts do not seem to extend from the PVM to the erythrocyte membrane neither as individual lamella nor via tubular connectors (Hanssen *et al.*, 2010).

It has been discussed whether nascent Maurer's clefts bud from the PVM already carrying all their cargo or if cargo is steadily delivered via vesicular trafficking to clefts in the cytosol of the erythrocyte. Today, controversial data exist from fluorescence recovery after photobleaching or photoconversion experiments about transport to Maurer's clefts. Photobleaching of a MAHRP1-GFP labelled Maurer's cleft did not result in fluorescence recovery (Spycher *et al.*, 2006) whereas Grüring *et al.* could show recovery after photoconversion of REX2-Dendra (personal communication (Grüring *et al.*, 2010)) indicating that new cargo must arrive at the clefts after initial budding from the PVM. However, it is possible that different proteins are differently transported to the Maurer's clefts and thus both observations might be correct. MAHRP1 might be a structural protein already involved in early assembly of Maurer's clefts, whilst REX2 might have a different function at a later stage and thus might be transported to Maurer's clefts in a vesicular way.

MAHRP1 was found in earlier stages than MAHRP2 indicating that tethers seem to be formed after the development of Maurer's clefts. MAHRP2 might be transported across the cytosol in a protein complex that then associates with these tubular structures. The formed tether might bind to a cleft and/or membrane initiating the tethering of the clefts. Whether lipids are involved in these processes also needs to be tested.

It has been suggested that during younger blood stages Maurer's clefts are motile and move around within the cell. Grüring and colleagues have claimed that this movement suddenly stops during trophozoite stages (Grüring *et al.*, 2010). Occurrence of MAHRP2 is also time dependent and occurs shortly before the suggested time point of Maurer's clefts arrest. Tethers could be a structural component to keep the clefts in close proximity to the membrane to facilitate

protein transport to the erythrocyte surface or to traffic proteins along these filament-like tethers. It is tempting to speculate that *Pf*EMP1 appearing on the surface about 24 hours post invasion (Horrocks *et al.*, 2005) whilst transiting through Maurer's clefts might be potential cargo for which such tethering could be essential. However, the process of fixation and its function remains elusive. The availability of a parasite clone which has a drastic reduction of MAHRP2 protein levels induced by fusion with the FKBP destabilizing domain allows to test whether this influences *Pf*EMP1 trafficking to the surface of the erythrocyte. We have shown that *Pf*EMP1 is exported beyond the parasite confines in these parasites but have not yet tested its surface exposure. We know, however, that the exposure of *Pf*EMP1 is not essential for parasite survival in culture.

Because we failed to knock out *mahrp2* and even because with the FKBP domain degradation system MAHRP2 is present in minute amounts we propose a rather essential role for the attachment of Maurer's clefts by tethers. Tethers could be involved or mediate the transport of proteins triggering egress or they might be involved in the formation of new permeability pathways. They even might play a role in nutrient uptake. Older parasite stages require more nutrients from the extra cellular space and tethers might generate a connection between the intra and extra cellular space.

Maurer's clefts definitely play an important role in protein transport to the erythrocyte membrane. Proteins destined for the erythrocyte membrane transiently associate with the clefts such as *Pf*EMP1, STEVOR, KAHRP, or *Pf*EMP3 (Knuepfer *et al.*, 2005b, Knuepfer *et al.*, 2005a, Lavazec *et al.*, 2006, Przyborski *et al.*, 2005, Wickham *et al.*, 2001). Furthermore, the Maurer's clefts resident proteins MAHRP1 (Spycher *et al.*, 2008) and SBP1 (Cooke *et al.*, 2006, Maier *et al.*, 2007) play an essential role in the trafficking of *Pf*EMP1 to the surface of the erythrocyte. In erythrocytes infected with *mahrp1* knock out parasites *Pf*EMP1 is not exported onto the surface of the erythrocytes nor transported to the Maurer's clefts. Maurer's clefts are still formed though they occur modified at an ultrastructural level. There are discrepancies in the phenotypes for the deletions of *sbp1* which have been generated in two different strains. In the 3D7 strain

*Pf*EMP1 was exported to the Maurer's clefts (Cooke *et al*., 2006) whilst it seemed to be arrested at the PVM in the CS2 strain (Maier *et al*., 2007). The exact role of MAHRP1 and SBP1 in *Pf*EMP1 export is not clear. They could interact directly or indirectly with *Pf*EMP1 which could be loaded into nascent Maurer's clefts and due to structural alterations of the clefts in knock out parasites this might be impossible. It could also be feasible that *Pf*EMP1 takes an alternative route and protein complex or vesicle formation is inhibited in the absence of MAHRP1 which could function as a chaperone at the PVM.

Maurer's clefts and tethers seem to be organelles unique to *P. falciparum*. Structures similar to Maurer's clefts have been observed in *P. vivax* where they are called Schüffner's dots (Wickert & Krohne, 2007). Orthologs of MAHRP1 and MAHRP2 have not been found in any other species. Recently, a culturing system and transfection technology has been established for *P. knowlesi* (Kocken *et al.*, 2002). It would be interesting to transfect these parasites with *mahrp1* and *mahrp2* to see where these proteins localize to and whether the proteins adopt a function in this species.

The importance of Maurer's clefts has been demonstrated in their essential role in the transport of the major virulence factor to the surface of the erythrocyte. A more sophisticated understanding of the function, composition, and interplay of Maurer's clefts and tethers could potentially lead to new possibilities to intervene with malaria.

Trafficking of the PEXEL-negative exported proteins MAHRP1 and MAHRP2

Parasite derived proteins involved in host cell refurbishment are secreted into the erythrocyte cytosol as well as exported to the surface of the red blood cell. No protein trafficking machinery is provided by the host cell and secretion across the various membranes appears highly complex. The parasite needs to establish its own intricate trafficking machinery. Most studies yet have focused on the export mechanism of proteins carrying a PEXEL motif. The PEXEL (Marti *et al*., 2004) or VTS (Hiller *et al*., 2004) motif is a pentameric motif with the consensus sequence

R/KxLxE/Q/D where x is any non-charged amino acid. PEXEL-positive proteins are processed in the ER (Boddey *et al.*, 2010, Boddey *et al.*, 2009, Chang *et al.*, 2008, Russo *et al.*, 2010) and thought to be secreted into the PV via vesicular traffic (Crabb *et al.*, 2010). In the PV they are recognized by the PVM translocon PTEX which mediates secretion into the erythrocyte cytosol (de Koning-Ward *et al.*, 2009). The discovery of the PEXEL motif allowed an *in silico* prediction of exported proteins based on the presence of this motif.

As exceptions to the rule, some Maurer's clefts resident proteins such as REX1, REX2, SBP1, and MAHRP1 as well as the tether protein MAHRP2 are exported beyond the PVM despite lacking a PEXEL motif or any classical signal sequence. Interestingly, all these proteins share some structural similarities in that they all contain a single transmembrane or hydrophobic domain. Is there an alternative pathway for PEXEL-negative proteins (PNEPs) of which many more might exist but fall through exportome predictions due to the absence of a predicted signal sequence?

It is unlikely that PEXEL-positive proteins and PNEPs share a common export mechanism. We must assume that PNEPs are not recognized by the translocon because mutation of a single amino acid of the pentameric PEXEL sequence abolishes translocation (Marti *et al.*, 2004) suggesting that the lack of such a motif also prevents this translocation. Co-translocation through a complex formed by PEXEL-positive proteins with PNEPs also occurs unlikely because it has been shown that PEXEL-positive proteins have to be unfolded prior to the translocation across the PVM (Gehde *et al.*, 2009). A complex of PEXEL-positive proteins and PNEPs would disintegrate upon unfolding and PNEPs would disperse in the PV and only PEXEL-positive proteins would be recognized by the translocon. The N-terminus of MAHRP2 displays in the N-terminus an LxE motif which resembles the processed N-terminus of a PEXEL motif (LxE/Q/D) that is recognized by the PTEX. This would offer the alternative explanation for a common export pathway in that PNEPs would be sorted differently and processed by another enzyme in the ER. However, mutation of the two amino acids did not impair export rendering this hypothesis rather unlikely.

We hypothesize that PNEPs are secreted completely differently from PEXEL carrying proteins. Analysing transfectants expressing truncated versions of MAHRP2 fused to the *gfp* reporter gene we found the central hydrophobic and N-terminal domain to be essential for guiding the protein to tethers. The C-terminal domain could be deleted and the protein was still exported in 3D7. Deletions within the N-terminal domain revealed that the first 15 amino acids and the histidine-rich central stretch are needed for translocation. Similar results were obtained in studies on REX2 in which the first 10 amino acids of the N-terminal domain and the transmembrane domain were sufficient to target REX2 to Maurer's clefts (Haase *et al.*, 2009). This, in contrast, is different for MAHRP1 or SBP1 where the second half of the N-terminus together with the transmembrane domain were sufficient for promoting export (Saridaki *et al.*, 2009, Spycher *et al.*, 2006). For REX1 the N-terminal hydrophobic stretch plus an additional 10 amino acids has been claimed to be sufficient to direct export while a coiled-coil region in the C-terminal domain was needed for binding to Maurer's clefts (Dixon *et al.*, 2008). So far, no common motif or perceptible sequence was found promoting the export of PNEPs.

From assays with the fungal metabolite brefeldin A (BFA) indicated that both MAHRP1 and MAHRP2 enter the classical secretion pathway as the addition of BFA redistributed the proteins to the ER. To further study the ER dependency of the export pathways of MAHRP1 and MAHRP2 we attached a signal peptide (SP) derived from the merozoite surface protein 1 (MSP1) N-terminally to the protein. Based on solubility analysis we expected MAHRP1 to insert into the ER membrane as an integral membrane protein similarly as we observed it without SP attachment. For MAHRP2, believed to naturally associate to the membrane peripherally facing the cytosol, we expected the attached SP to guide the protein into the ER lumen whereby the hydrophobic domain of MAHRP2 would act as a membrane retention signal. Thus, the topology of MAHRP1 would not be changed compared to the wild type protein. On the other hand, MAHRP2 would no longer be peripherally associated but integrally inserted into the membrane. In addition, we generated constructs with a signal peptide but lacking the transmembrane or

hydrophobic domain which then should have lead to soluble proteins completely translated into the ER lumen.

The addition of a signal peptide did not affect export of both full length proteins again indicating an ER-dependent export. Surprisingly, MAHRP1 with a signal peptide but without its transmembrane domain (SP-M1ΔTM) was exported to Maurer's clefts. However, light microscopy does not allow to test the location of MAHRP1 at the Maurer's clefts and MAHRP1 might be present in the lumen of the clefts only. Solubilization assays could shed light on the characteristics of the SP-M1ΔTM protein. MAHRP2 without the hydrophobic stretch but with signal peptide (SP-M2ΔTM) was not translocated across the PVM. This is similar to REX2, in that the fusion protein with a signal peptide derived from ETRAMP10.1 was also exported even though not as efficient as MAHRP2 with the MSP1 signal peptide (Haase *et al*., 2009). REX2 fusion protein lacking the transmembrane domain was found accumulated at the PV (Haase *et al*., 2009) similarly to the situation with SP-M2ΔTM. These differences indicate that MAHRP1 might take a different export pathway than MAHRP2 and possibly REX2. This hypothesis is also supported by the fact that truncation of the first 12 N-terminal amino acids abolished export of MAHRP2 (similarly to REX2 (Haase *et al*., 2009)) whilst the deletion of the first 51 amino acids of MAHRP1 did not influence export (Spycher *et al*., 2006).

Based on these findings we suggest a vesicular mediated trafficking for MAHRP1 to the Maurer's clefts (Fig. 1, upper panel). Our findings would as well support a similar vesicular transport for MAHRP2 (Fig. 1, lower panel) except that SP-M2ΔTM is not exported. Furthermore, we could enrich mobile MAHRP2-labelled tethers by ultracentrifugation but no labelled vesicles. MAHRP2 might be exported to the PVM also by vesicular transport but from there onward we propose a different means of transport (Fig. 2). Membrane association seems to be essential for translocation across the PVM for MAHRP2 but not for MAHRP1. Although, apart from the hydrophobic domain, all the requirements for MAHRP2 export lies in the N-terminal domain which is present in the soluble SP-M2ΔTM, it is not exported across the PVM unlike SP-M1ΔTM. SP-M1ΔTM is most probably recognized via the N-terminal domain and packed as soluble protein into vesicles destined for

Maurer's clefts. MAHRP2 could associate with other membrane associated proteins at the PVM possibly via the histidine-rich stretch inducing protein complex formation. This complex formation could perhaps also include soluble proteins from the erythrocyte cytosol. The assembled complex might then be released from the membrane to traffic across the erythrocyte cytosol to tethers (Fig. 2, upper panel). It could also be possible that tethers are formed upon fusion of several of these complexes in the cytosol.

The membrane associated complex assembly at the PVM might be facilitated by further membrane association mediated by the predicted N-terminal amphipathic helix (Fig. 2, lower panel). Amphipathic helices can mediate membrane targeting whereby the non polar side of the helix is embedded in the membrane. The requirement of an N-terminal membrane association which is facilitated through the peripheral membrane association via the hydrophobic domain for protein complex assembly could explain why soluble SP-M2ΔTM is not exported. It would be interesting to mutate amino acids destroying the amphipathic character to see if export is impaired. Further experiments are needed to get more evidence on the alternative mechanisms of PNEP mediated export.

In this thesis we tried to characterize two early expressed proteins of *P. falciparum*, MAHRP1 and MAHRP2. Exported into the erythrocyte cytosol they both are involved in host cell refurbishment localizing to organelles unique to *P. falciparum*. Even though rather similar in structure they localize to different organelles in the erythrocyte cytosol and seem to be exported by different mechanisms. The essential role of MAHRP1 in the trafficking of the major virulence factor to the surface of the erythrocyte confirms the importance to study proteins involved in host cell refurbishment to get a better understanding of the parasite's survival strategies.

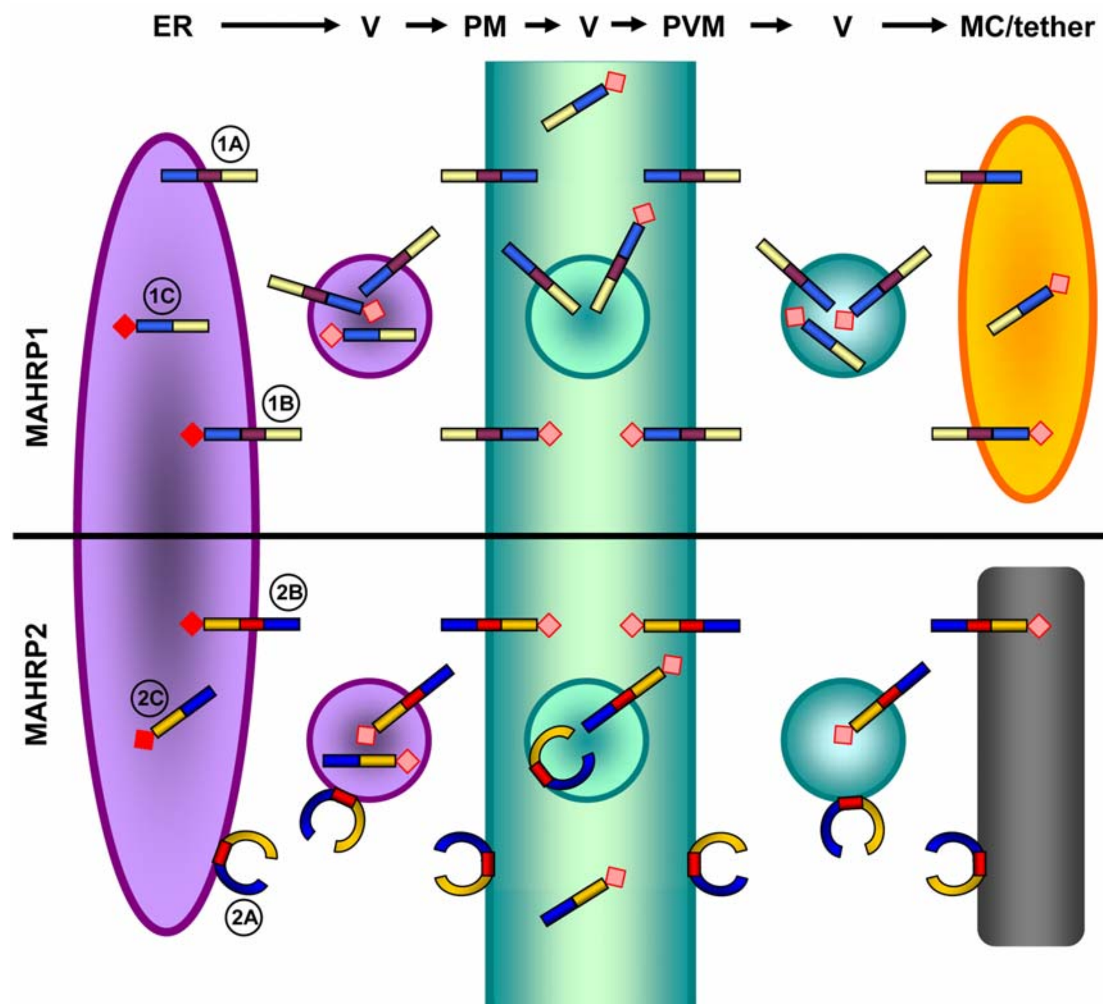


Figure 1. Schematic representation of potential vesicular trafficking pathways for MAHRP1 and MAHRP2. Three different versions of the protein are depicted namely wild type protein (1A, 2A), full length protein with an N-terminally attached signal peptide derived from MSP1 (1B, 2B), and protein lacking the transmembrane or hydrophobic domain with an N-terminally attached signal peptide (1C, 2C).

MAHRP1 and MAHRP2 are secreted ER-dependently. On the basis of solubility characteristics it is hypothesized that MAHRP1 enters the ER as an integral membrane protein (1A) whereas MAHRP2 would associate peripherally with the ER membrane (2A). The attachment of an N-terminal signal peptide should guide both proteins into the lumen of the ER whereby the transmembrane as well as the hydrophobic domain should act as a membrane retention signal leading to an integral membrane insertion of both proteins (1B, 2B). Lacking the

transmembrane or hydrophobic domain the signal peptide is expected to guide both proteins into the ER lumen as the retention signal is lost (1C, 2C). Secretion from the ER to the parasite membrane (PM) is thought to occur via vesicular trafficking. Proteins are packed into vesicles either as integral, membrane associated, or soluble proteins. Upon fusion with the PM the N-termini of integral MAHRP1 (1A, B) and MAHRP2 (2B) face the parasitophorous vacuole (PV). Soluble proteins (1C and 2C) are released into the PV and wild type MAHRP2 (2A) peripherally associates with the PM facing the parasite cytosol. Further translocation across the PV again is indicated by vesicular transport whereby integral MAHRPs are inserted into the PV membrane (PVM) facing the PV with their N-termini (1A, B and 2B). Peripheral PV membrane associated MAHRP2 (2A) faces the cytosol of the erythrocyte. Here we show hypothetical the vesicular traffic from the PVM to Maurer's clefts or tethers whereupon integral membrane proteins (1A, B and 2B) are inserted into Maurer's clefts (MC) or tethers facing the lumen with their N-termini. Wild type MAHRP2 is expected to peripherally associate with tethers facing the cytosol of the erythrocyte if membranous structures are present (2A). Soluble proteins are released into the MC lumen (1C) whereas no lumen has been demonstrated in tethers. All current experiments would support this hypothesis except that soluble SP-M2ΔTM (2C) is not secreted from the PV.

MAHRP1 (upper panel): N-terminal domain is displayed in blue, the C-terminal domain in light yellow, and the transmembrane domain in purple. MAHRP2 (lower panel): N-terminal domain is displayed in yellow, the C-terminal domain in blue, and the hydrophobic domain in red. The signal peptide from MSP1 which was N-terminally attached is indicated in red in the ER and after ER exit in pink allowing differentiation of the different constructs. ER, endoplasmic reticulum; V, vesicle, PM, parasite membrane; PV, parasitophorous vacuole; PVM parasitophorous vacuolar membrane; MC, Maurer's clefts.

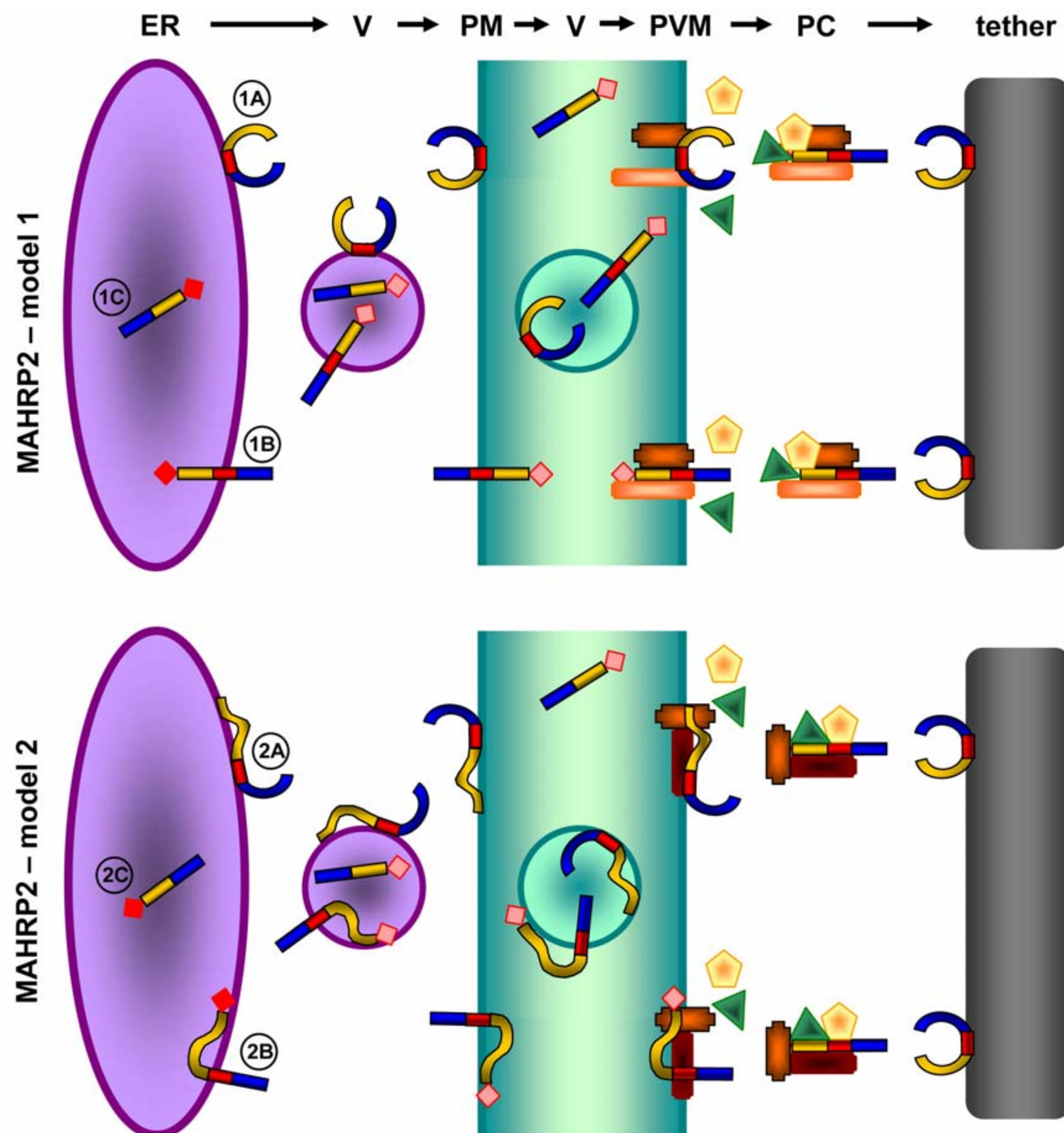


Figure 2. Schematic representation of hypothetical trafficking pathways for MAHRP2 including a translocation across the erythrocyte cytosol in a protein complex. Three different versions of the protein are depicted namely wild type protein (1A, 2A), full length protein with an N-terminally attached signal peptide derived from MSP1 (1B, 2B), and protein lacking the transmembrane or hydrophobic domain with an N-terminally attached signal peptide (1C, 2C).

In model 1 (upper panel), proteins are trafficked to the PVM as explained in figure 1. At the PVM, membrane associated (1A) and integral (1B) MAHRP2 bind to other membrane associated proteins initiating a protein complex formation which possibly could also involve soluble proteins and chaperones from the

erythrocyte cytosol. The assembled protein complex is then released from the PVM and diffuses through the cytosol of the erythrocyte until it docks with tethers or associates with other complexes forming tethers. A similar scenario is described in model 2 (lower panel). In this model the membrane binding capability of the potential N-terminal amphipathic helix is taken into account. The requirement of an N-terminal membrane association which is facilitated through the peripheral membrane association via the hydrophobic domain for protein complex assembly could explain why soluble SP-M2ΔTM is not exported. The importance of the N-terminal domain in export of the protein has been confirmed by parasite transfections with truncated version of MAHRP2. The assembled protein complex is then released from the PVM and diffuses through the cytosol of the erythrocyte until it docks with tethers or associates with other complexes forming tethers as in model 1. We currently have no experimental data falsifying model 2.

MAHRP2: N-terminal domain is displayed in yellow, the C-terminal domain in blue, and the hydrophobic domain in red. The signal peptide from MSP1 which was N-terminally attached is indicated in red in the ER and after ER exit in pink allowing differentiation of the different constructs. ER, endoplasmic reticulum; V, vesicle; PM, parasite membrane; PV, parasitophorous vacuole; PVM parasitophorous vacuolar membrane; PC, protein complex.

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Outlook

This thesis contributes to a better understanding of the biology of the malaria parasite *P. falciparum*. To verify our hypotheses and to further elucidate the function of tethers and Maurer's clefts as well as deciphering the export mechanisms of PNEPs I suggest the following experiments.

- The role of MAHRP1 in *Pf*EMP1 export is currently investigated. MAHRP1 knock out parasites are complemented with truncated versions of MAHRP1 known to be exported. Erythrocytes infected with reconstituted parasites are checked for *Pf*EMP1 surface exposure to identify the sequences of MAHRP1 required to promote *Pf*EMP1 export.
- The fusion of MAHRP2 with the destabilizing domain FKBP resulted in almost complete loss of the MAHRP2 protein in these transfected parasites.
  These 'knock down' transfectants should be analysed for several possible phenotypic changes. Some are listed here.
  - Trypsin cleavage and CD36 binding assays should demonstrate if *Pf*EMP1 is exposed on the surface of erythrocytes infected with MAHRP2 'knock down' parasites.
  - Do Maurer's clefts stop moving at trophozoite stage as in erythrocytes infected with wild type parasites? This can be approached by time-lapse imaging of live infected erythrocytes labelled with the lipid probe BODIPY-ceramide or of 'knock down' parasites transfected with a plasmid expressing MAHRP1-GFP.
  - Structural differences compared to erythrocytes infected with wild type parasites should be revealed by electron microscopy of thin sections.
  - Preliminary experiments indicated a reduced sensitivity of erythrocytes infected with mature 'knock down' parasites to sorbitol lysis compared to mature wild type parasites. Haemolysis

experiments should be performed to check whether the permeability of the infected erythrocyte membrane is altered.

- Cryo-electron microscopy of purified tethers enriched by differential ultracentrifugation should give better resolution and more information on the structure of tethers. To test whether lipids are involved in tether formation chloroform extraction should be performed followed by mass spectrometry analysis.
- Description of the 'tether proteome' needs additional purification steps of the enriched tether fraction to improve mass spectrometry analyses. This should be accomplishable by immunoprecipitation of whole tether structures. Present protein interaction candidates of MAHRP2 need to be verified for true interactions *in vitro*.
- To test whether clathrin is found in the cytosol of the erythrocyte, it should be tagged with an HA- or GFP-tag as the antibody against the existing TY-tag cross reacted with parasite proteins in immunofluorescence assays.
- Disruption of the amphipathic character of the predicted N-terminal helix of MAHRP2 by mutation of a few amino acids should reveal if additional membrane association of the N-terminal domain is essential for translocation of MAHRP2 across the parasitophorous vacuolar membrane.
- To test whether the first 20 amino acids of the MAHRP2 N-terminus carry trafficking information similar to the classical signal peptide of MSP1 (used in trafficking studies) or if they comprise additional information, the first 20 amino acids of MAHRP2 should be replaced by the MSP1 signal peptide, and export of the fusion protein should be checked.
- To investigate if SP-M2-GFP really is an integral membrane protein in the ER, if SPM1ΔTM-GFP is truly found soluble in the Maurer's cleft lumen, and if $M1_{M2TM}$-GFP orientation is inverted in Maurer's clefts compared to wild type MAHRP1, solubility and Proteinase K protection assays should be performed.

MAHRP1 knock out parasites: Single clone generation by limited dilution

Methods

Limited dilution

Subsequent to the successful generation of parasites after transfection with pTK_M1 disrupting the *mahrp1* gene via 5' single crossover, parasites were cloned by limiting dilution at 10, 1, and 0.1 parasites per well in a 96-well plate. Parasitemia was counted by Giemsa-stained smears, and parasites were diluted in RPMI medium, such that there were 10, 1 or 0.1 parasites per µl. For each plate a cell suspension was prepared for 80 wells (16 mL). 80 µl of the 3 different parasite dilutions were mixed with 15.6 mL RPMI medium, 8 µl WR, and 320 µl fresh blood to give a hematocrit of 2%. 200 µl of each of these mixtures were added to 60 wells of a 96-well plate to obtain a final dilution of 10, 1, or 0.1 parasites per well at 2% hematocrit. Outer wells were filled with 200 µl medium. The culture medium was changed and 0.4% hematocrit added on day 7 and day 14. On day 14 and 21 the status of each well was checked with thick blood smears stained with Giemsa. The content of wells examined harbouring parasites was transferred to 6-well plates, RPMI medium and blood was added to give volume of 5 mL at a hematocrit of 5%.

Southern Blot

To verify successful knock out clone selection and 5′ single crossover, gDNA from 3D7 wild-type parasites and MAHRP1 knock out clones was digested with TspRI and MluI according to previous experiments (Spycher *et al.*, 2008) and analysed by Southern blot. The blot was probed with a ^{32}P-dATP-labelled *h*dhfr fragment.

Western Blot

A saponin-lysed parasite pellet was resuspended in Laemmli sample buffer, separated on a 12.5% acrylamide gel, and blotted to nitrocellulose (Hybond-C extra; GE Healthcare) for 1.5 h using a Trans-Blot semi-dry electroblotter (Bio-Rad). The nitrocellulose membrane was blocked in 8% skim milk, 0.1% Tween in Tris-buffer. The antibodies used were: mouse anti-MAHRP1 (1:1000), monoclonal

mouse anti-GAPDH (1:5000) (Daubenberger *et al.*, 2003), horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, 1:20 000).

CD 36 binding assay

Recombinant human CD36 (125 mg/ml in PBS) or 1% BSA in PBS (control) was immobilized on a Petri dish as described previously (Spycher et al., 2008). Dishes were incubated in a humid chamber over night at 4°C. Non-specific binding was blocked with 1% BSA in PBS for 30 min at room temperature and washed with RPMI-Hepes. Infected erythrocytes in RPMI-Hepes and 10% human serum (5% hematocrit) were added and incubated for 30 min at 37°C. The dish was gently washed with RPMI-Hepes. Bound cells were fixed with 2% glutaraldehyde in RPMI-Hepes for 2 h and stained with 10% Giemsa for 10 min. Bound infected RBCs were quantified in 8 or 10 different 0.2 mm^2 areas. The mean value was calculated as infected RBCs per mm^2 and normalized to a parasitemia of 1%. The experiment was performed three times.

Results

After limited dilution and analysis by Southern blot, Western blot, and CD36 binding assays, two clones (clones 7 and 9) could be selected with correct 5' single crossover of the plasmid pTK_M1 that leads to the disruption of the *mahrp1* gene.

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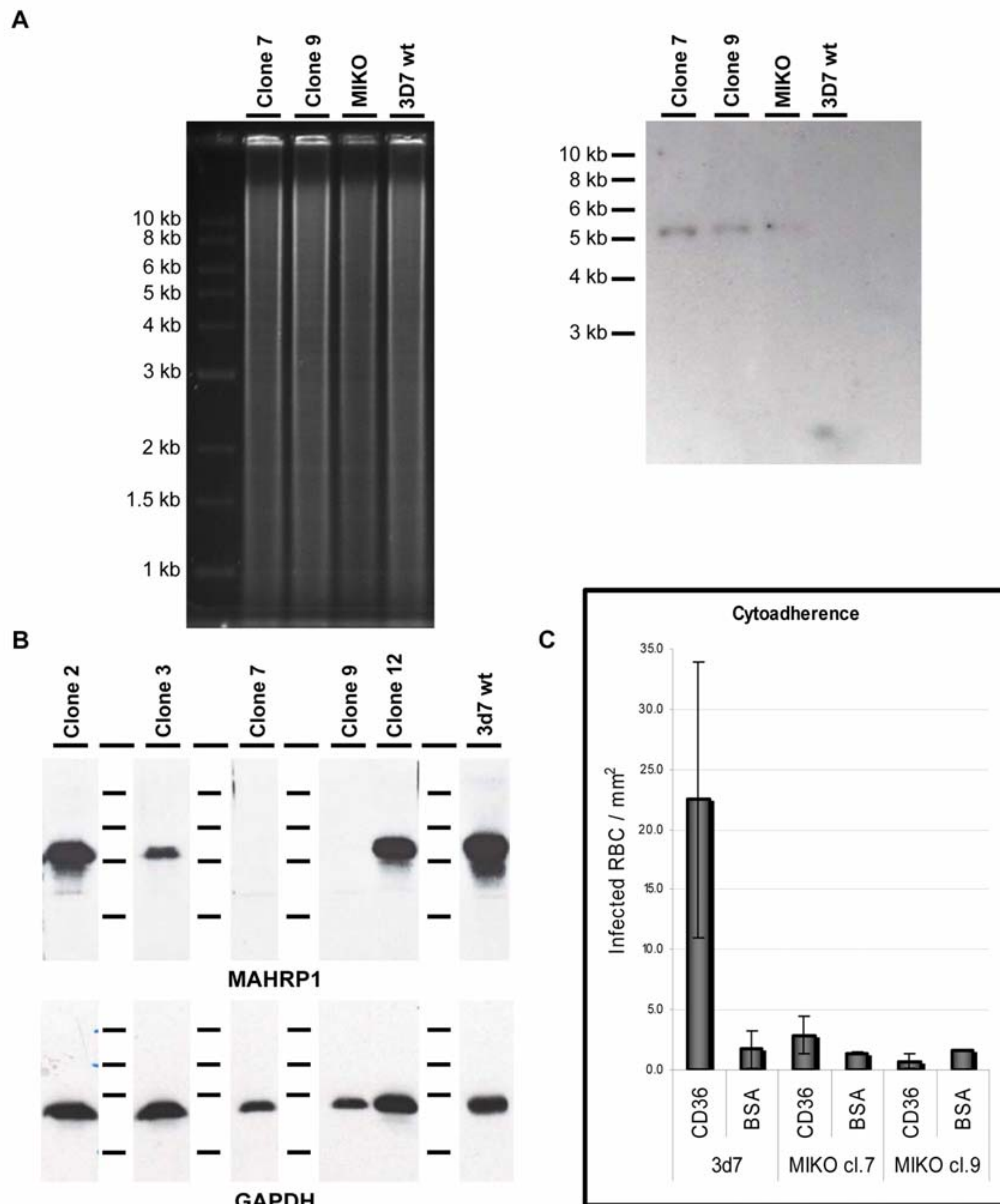

Figure 1: (A) Southern blot analysis confirms selection of clones with integrated pTK_M1 into the MAHRP1 locus. Digested genomic DNA (left panel) was probed with a labelled *h*dhfr probe (right panel). The predicted sizes are 5333 (5' single crossover), 7565 (3' single crossover), 4767 bp (double crossover). No band is observed in wild type gDNA. (B) Western blot analysis of saponin-insoluble material from erythrocytes infected with *Pf*3D7ΔM1 transfectant clones and wild type 3D7 probed with mouse anti-MAHRP1 (upper panel) or mouse anti-GAPDH (lower panel) antibodies. (C) Bar graph depicting differential binding to CD36 of

erythrocytes infected with *Pf*3D7ΔM1 clones or wild-type 3D7. CD36 (or BSA as control) was immobilized onto plastic Petri dishes. Bound infected RBCs were quantified in 10 different areas (0.2 mm^2). The mean value was calculated as infected RBCs per mm^2 and normalized to a parasitaemia of 1%.

Curriculum Vitae

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"Characterization of two Plasmodium falciparum *proteins, MAHRP1 and MAHRP2, involved in host cell refurbishment"*

2005 – 2006 **Master thesis in molecular biology** at Pediatric Immunology, Department of Biomedicine, University of Basel and University Children's Hospital, Basel, Switzerland
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Publications

MAHRP2, an exported protein of *Plasmodium falciparum*, is an essential component of Maurer's cleft tethers. Pachlatko E, Rusch S, Müller A, Hemphill A, Tilley L, Hanssen E, Beck HP. Mol Microbiol. 2010 Jul 6. [Epub ahead of print]

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Conferences

2009 **7. Malariatreffen der Paul Ehrlich Gesellschaft (PEG) und der Deutschen Gesellschaft für Tropenmedizin und Internationale Gesundheit (DTG):** Hamburg, Germany

Talk: *"A novel exported protein in* Plasmodium falciparum *localizes to recently described tethers"*

Molecular Parasitology Meeting: Woods Hole, USA

Talk: *"A novel exported protein in* Plasmodium falciparum *localizes to recently described tethers"*

BioMalPar Conference: Heidelberg, Germany

Poster: *"Trafficking of a novel histidine-rich protein to new structures in the cytosol of red blood cells infected with* P. falciparum*"*

PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology (SSTMP): Basel, Switzerland

Talk: *"A novel exported protein in* Plasmodium falciparum *localizes to recently described tethers"*

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Talk: *"The Maurer's cleft protein MAHRP1 is essential for trafficking of* Pf*EMP1 to the surface of* Plasmodium falciparum *infected erythrocytes"*

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Talk: *"Localization and functional analysis of the* Plasmodium falciparum *membrane associated histidine-rich proteins 1 and 2 (MAHRP-1 and -2)"*

PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology (SSTMP): Münchenwiler, Switzerland

Talk: *"Localization and functional analysis of the* Plasmodium falciparum *membrane associated histidine-rich proteins 1 and 2 (MAHRP1 and -2)"*

Joint meeting of the Swiss Society of Tropical Medicine and Parasitology and the French Society and German Society of Parasitology: Strasbourg, France

Poster: *"MAHRP-2, a* P. falciparum *histidine-rich protein with potential role in trafficking of parasite derived proteins"*

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