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Expression of dominant negative mutant proteins to interfere with *Plasmodium* liver stage development

Dissertation

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1 Hypothesis and aims

As *Plasmodium* parasites develop intracellularly, interactions between host cell and parasite are mandatory. In addition, the *Plasmodium* liver stage is clinically silent and precedes the pathogenic blood stage. These two facts make the interaction between host cell and parasite within the liver an excellent target for intervention, but so far, our knowledge of the molecular details of this interaction is insufficient. More research on this topic is necessary. This thesis concentrates on parasite protein transport during the *Plasmodium* liver stage. The protein Sar1 GTPase is involved in intracellular protein transport. Genetically modified parasites of the rodent model *Plasmodium berghei* were used to infect and analyse hepatoma cells *in vitro* and their development analysed. A liver stage-specific promotor was used to express a dominant negative mutant form of the protein Sar1 GTPase exclusively during the liver stage. This thesis investigates the role of intracellular protein transport during the development of the parasites inside the hepatocyte.

2 Introduction

2.1 Malaria

Malaria is an infectious disease caused by a protozoan parasite of the genus *Plasmodium*. It has a complex life cycle involving an *Anopheles* mosquito, where its sexual reproduction takes place and a vertebrate as its intermediate host.

There are five parasite species that affect humans:

- *Plasmodium falciparum* is the most common and causes the disease malaria tropica. It is responsible for most malaria deaths.
- *Plasmodium vivax* is the most widespread and can form a dormant liver stage. It causes malaria tertiana.
- *Plasmodium ovale* also causes malaria tertiana.
- *Plasmodium malariae* causes the disease malaria quartana.
- *Plasmodium knowlesi* usually causes malaria among monkeys and was recorded in recent years to also infect humans.

Other species affect other mammals, reptiles and birds.

Epidemiology

Most of the world's malaria infections occur in the African Region. In 2013, there were 198 million diagnosed cases of malaria infection and 584,000 deaths (WHO, 2014).

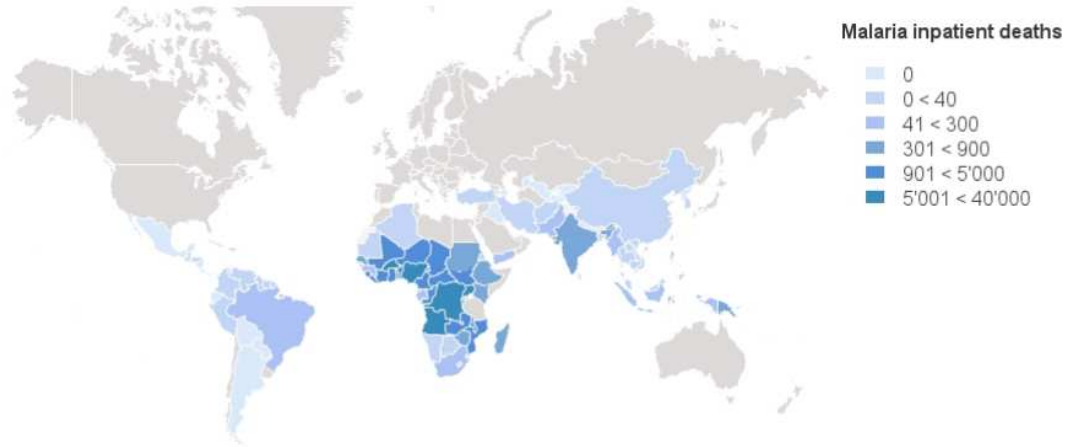

Figure 2.1.1: Malaria inpatient deaths (©2015 - Global Malaria Mapper)
Malaria mortality rates per country are shown in different shades of blue. Dark blue represents high mortality rates and light blue indicates low mortality rates (see legend). Countries labelled in grey have no applicable data. Data is collected for inpatients only.

Due to poor medical and hygiene standards, as well as problems such as malnutrition, African people suffer from comorbidity and poor levels of resistance. Additionally, these countries have the least access to prevention, diagnosis and treatment. Thus 90 % of malaria deaths occur in the African Region (WHO, 2014). The vast majority of cases affect children under the age of 5 years, as adults living in malaria endemic regions typically develop semi-immunity against symptomatic disease.

2.1.1 Clinical aspects

Different species of *Plasmodium* cause different forms of malaria. As *P. falciparum* is responsible for most infections and malaria deaths each year, the following description is focused on Malaria tropica, the disease caused by *P. falciparum*:

It shows no typical fever pattern and is characterised by high parasitaemia, anaemia and is often combined with neurological complications. The incubation time is between 6 days and 6 weeks (DTG, 2014).

Symptoms and pathophysiology

Once *Plasmodium* parasites have reached the blood stage (see chapter 2.2), symptoms of malaria set in:

Within the infected red blood cells, merozoite proliferation occurs. When this is complete, the erythrocyte bursts and releases merozoites and waste products. The liberation of toxins induces inflammatory cytokine release, thus triggering the fever reaction. Besides fever, many of the first symptoms are similar to the flu: headache, body pain, chills and vomiting (Murphy et al., 2006). High parasitaemia means many bursting red blood cells, thereby causing severe anemia. *P. falciparum* is characterised by its ability to cause adherence of red blood cells to blood vessel endothelia (Jensen et al., 2004), which leads to a sequestration of erythrocytes. Sequestration impairs perfusion; the hereby-resulting hypoxia can cause cerebral malaria and multi-organ failure (Idro et al., 2010).

Diagnosis

Patients with typical symptoms and exposure to *Anopheles* mosquitoes should be tested for malaria:

The most important and least expensive tool is the microscopy of a thin or thick blood smear stained with “Giemsa solution”. This allows not only identification

of parasites but also the direct differentiation between the different species as well as the determination of the parasitaemia. 50 parasites per microlitre of blood is the detection limit of microscopy and this correlates with a parasitaemia of under 0.001 % (DTG, 2014). In addition, there are immunological tests such as the "rapid diagnostic test", which detects parasite *Plasmodium* antigen, as well as the detection via a polymerase chain reaction (PCR). These methods are more expensive than blood smearing.

2.1.2 Strategies against malaria

In the face of the high morbidity and mortality of malaria, several strategies were developed to fight against this disease. There are different drugs available for treatment, as well as both mechanical and chemical methods of prohibiting mosquito biting. Increasing resistance to both chemo-preventative and therapeutic drugs is a problem and makes these methods insecure. Research is ongoing to develop new drugs as well as a vaccine against the disease.

Prophylaxis and therapy

Anopheles mosquitoes bite mainly between dusk and dawn. Thus insecticide-treated mosquito bed nets are a very effective strategy for preventing bites from infected mosquitoes (WHO, 2014). Another method is indoor residual spraying of insecticides, which kills mosquitoes inside buildings, but on the other hand, stimulates insecticide resistance.

So-called chemoprevention, as opposed to treatment of symptomatic infection, is only recommended for pregnant women and young children (WHO, 2014): sulfadoxine-pyrimethamine and amodiaquine can be used. For travellers, the "Deutsche Gesellschaft für Tropenmedizin" recommends Atovaquone/Proguanil (Malarone®), Doxycycline (off-label use) or Mefloquine (Lariam®).

For treatment, again different drugs are available for different clinical situations (WHO, 2014; DTG, 2014): chloroquine, artemisinin-based combination therapy, primaquine and quinine with the possibility to combine with tetracycline antibiotics.

Vaccination approaches

The pre-erythrocytic stage is clinically silent and precedes the pathogenic blood stage and is therefore a convenient target for vaccines and medical prophylaxis against malaria. In 1967, Nussenzweig *et al.* immunised mice with radiation-attenuated sporozoites of *P. berghei* and showed that a large percentage of these animals were protected against challenge with viable sporozoites (Nussenzweig et al., 1967). With this strategy, Hoffmann *et al.* immunised humans quite successfully, with a protection of 94 % (Hoffman et al., 2002). One large problem remains the clinical and logistical implementation of the vaccination strategy; immunising large numbers of susceptible persons with irradiated sporozoites. It is not routinely possible to culture sporozoites, meaning that large numbers of *P. falciparum*-infected mosquitoes need to be produced and dissected and the sporozoites stored under conditions that maintain viability. The development of a subunit-vaccine therefore seemed an auspicious idea because it would be far simpler and cheaper to generate and store material for vaccination (Seder et al., 2013). The most prominent candidate is the RTS,S vaccine, containing circumsporozoite protein (CSP) epitopes, that provide targets for both antibody and cellular responses, fused to hepatitis B surface antigen (HBsAg) together with unfused HBsAg (Stoute et al., 1997). In field trials, however, the efficacy against clinical malaria is only 65.2 % (Abdulla et al., 2008) and the vaccine has failed compared to the degree of protection and long-lasting immunity observed with the irradiated sporozoite vaccine. Although other antigens expressed by irradiated sporozoites have been studied as vaccine targets, we do not know the complete antigenic repertoire seen

by the immune system following immunization with the irradiated sporozoite vaccine that protects people from malaria (Hoffman and Doolan, 2000). It is essential that we increase our understanding of the mammalian response to whole-sporozoite immunisation.

Genetically attenuated parasites

A promising approach for both the development of a whole-parasite vaccine and for studies to better understand immune responses against whole sporozoites is the generation of genetically attenuated parasites, that are able to infect liver cells but not red blood cells and therefore will not cause clinical symptoms of malaria. As genetic manipulation of *Plasmodium* is performed during the blood stage, the viability of the modified parasite before reaching the liver stage is essential for use as a vaccine (Combe et al., 2009), otherwise the parasite cannot proliferate in the blood or mosquito and therefore cannot form sporozoites to infect hepatocytes or hepatoma cells. A simple knock-out is only feasible if the gene is essential in the liver stage but not essential in any other stages, as is true for the "upregulated in infectious sporozoites 3" (UIS3) and UIS4 genes (Mueller et al., 2005).

As an alternative approach, parasites can be attenuated by expression of a dominant negative mutant protein. When a mutant protein can interfere with the function of the endogenous protein and acts dominantly over the wild-type gene product, it is called a dominant negative mutant protein. For this, the expression of such proteins must be driven by a strong promoter and the mutated gene product must still interact with at least some of the same elements as the wild-type product, but block some aspects of its function. In *Plasmodium* parasites, such dominant negative mutant proteins can be used for stage-specific interference, using a stage-specific promoter. In *P. berghei*, a liver stage-specific promoter, called lisp2, was recently identified. Helm *et al.* and De Niz *et al.* showed the lisp2 (formerly called LSA4) promoter to be silent in the blood and mosquito stages and to have

an increasing activity in the liver stage from 24 hours post infection (hpi) onwards, with a maximum at 54 hpi (Helm et al., 2010; DeNiz et al., 2015). There is great promise, therefore, in using stage-specific protein expression for interference with parasite development at specific life cycle stages, in particular for the analysis of, and targeting of, the *Plasmodium* liver stage (Nagel et al., 2013). The expression of genes under the control of the lisp2 promoter would therefore make it possible to interfere only with parasite development during the liver stage, and is therefore a great tool for immunisation studies.

2.2 Life cycle of *Plasmodium*

Plasmodium is a parasite of the phylum *Apicomplexa*, most of which are obligate intracellular protozoan parasites. The life cycle of *Plasmodium* parasites can be separated into three stages: the mosquito stage within an *Anopheles* mosquito and the liver and blood stage, both within its vertebrate host.

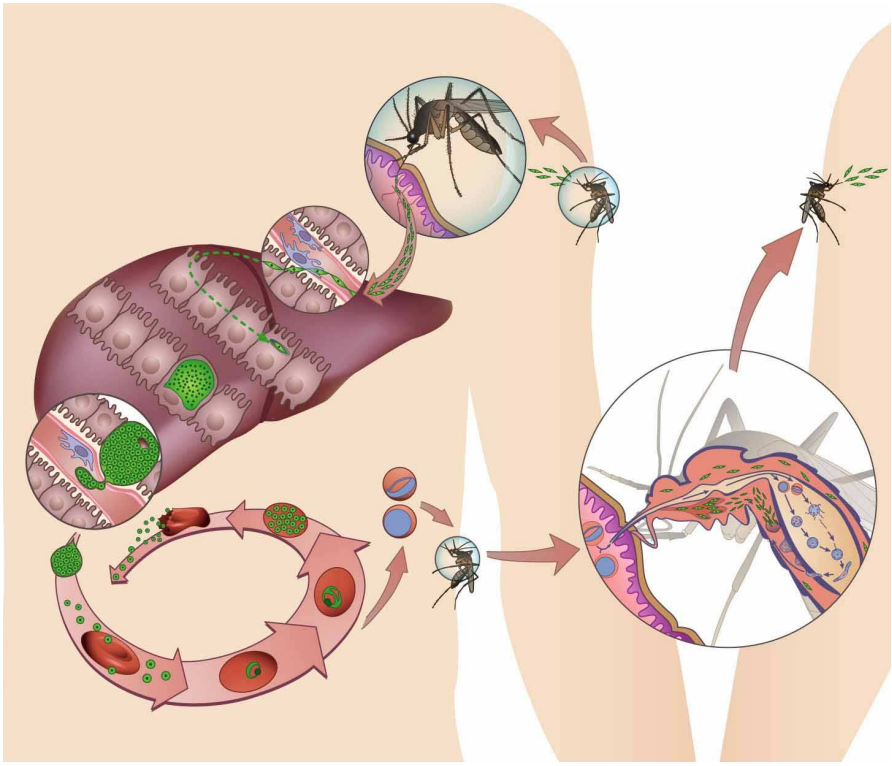

Figure 2.2.1: The *Plasmodium* life cycle (Sturm and Heussler, 2007)
Plasmodium parasites are transferred to the dermis of a human when an infected *Anopheles* mosquito bites and delivers sporozoites. From the dermis, they invade blood vessels and follow the bloodstream to the liver, where they migrate through several cells until they infect finally one hepatocyte. After a huge replication step, the PVM ruptures and the host cell is filled with merozoites, which pinch off in small packages, the merosomes, into the bloodstream. Within the bloodstream, merosomes burst, liberate merozoites, infect red blood cells and start an asexual reprodution cycle. Some merozoites develop into gametocytes, the sexual form of *Plasmodium*. These can be taken up by a mosquito during blood feeding. First inside, then outside the mosquito's midgut, the sexual reproduction occurs, completing the whole life cycle with the sporozoites making their way to the salivary glands.

2.2.1 Blood stage

The red blood cell-infectious form of *Plasmodium* parasites is called the merozoite, which contacts erythrocytes: On the merozoite surface, proteins such as "merozoite surface protein 1" (MSP1) allow them to attach to potential host cells (Herrera et al., 1993; Kauth et al., 2003), followed by a movement and re-orientation of the merozoite and deformation of the red blood cell surface. Finally the parasite enters the erythrocyte (Cowman et al., 2012), where it lives and grows inside a cavity, the so-called parasitophorous vacuole (PV). The invasion is followed by an asexual replication cycle, where the parasite passes through several developmental stages:

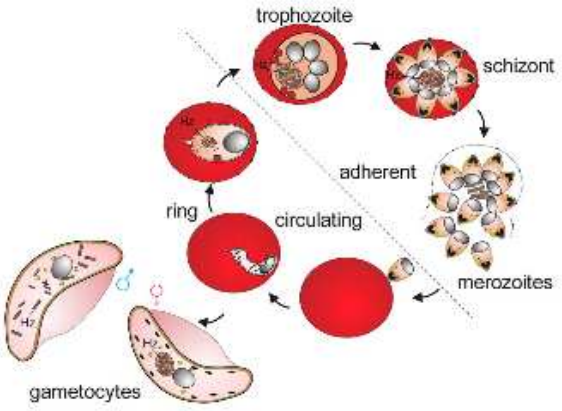

Figure 2.2.2: The blood stage of *Plasmodium* parasites (Khoshmanesh *et al.*, 2014)
The blood stage is initiated when one merozoite invades a red blood cell within the blood stream. The first phase is called the ring stage, characterised by metabolism of hemoglobin, leading to the accumulation of hemozoin (Hz). During the trophozoite stage, the parasite increases in size, organelles replicate and DNA is synthesised, but no nuclear division occurs. Finally the schizont stage is formed, where nuclear division occurs, the plasma membrane ultimately invaginates and merozoites are formed. When the red blood cell bursts, merozoites are released into the blood stream and the cycle starts again. Mature stage parasites adhere to the endothelium and are seen rarely in blood smears. Some merozoites develop into male and female gametocytes, the sexual form of *Plasmodium*. Mature gametocytes are found in the blood stream and can be observed in a blood smear.

The first phase is the ring stage, characterised by metabolism of hemoglobin and secretion of proteins to adjust the red blood cell. The parasite ingests hemoglobin, which is transported to, and degraded in, an acidic food vacuole (Rosenthal and Meshnick, 1996). Here, the toxic heme is converted to non-toxic hemozoin (Dasari and Bhakdi, 2012) and globin is hydrolysed to free amino acids (Rosenthal and Meshnick, 1996). The next stage is called the trophozoite. This mononuclear parasite grows and synthesises DNA without nuclear division, developing into the schizont stage, where nuclear division occurs (Tuteja, 2007). This multinucleated schizont invaginates its membrane and forms 16-32 daughter cells, resulting in even more merozoites to again invade red blood cells. When the parasitophorous vacuole membrane and the host cell membrane break down, merozoites are released into the blood stream and start invading other erythrocytes.

Most invading merozoites replicated to again produce more merozoites, whereas some of them finally become gametocytes, the sexual form of *Plasmodium*. Male and female gametocytes circulate within the blood stream, where they can be taken up by a mosquito with the next blood meal.

2.2.2 Mosquito stage

Anopheles mosquitoes serve as the definitive host of *Plasmodium* parasites; here the sexual reproduction occurs. The sexual forms of *Plasmodium*, the *gametocytes*, enter the mosquito's midgut during a blood meal, where gametogenesis is initiated (Aly et al., 2009): The male microgametocyte matures to 8 microgametes, which undergo so-called exflagellation in being released from the red blood cell; the female macrogametocyte matures to a macrogamete. The fusion of a male and a female gamete, the fertilization, forms a zygote, which transforms into the ookinete, an elongated, motile cell. After traversal of several midgut epithelial cells, the ookinete exits the midgut and transforms into an oocyst. Subsequently an asexual

reproduction step called sporogony forms thousands of sporozoites, which egress from the oocyst to make their way in the hemolymph to reach the salivary glands (Aly et al., 2009). The whole process takes 8-16 days, depending on the species and the environmental temperature. Mature sporozoites are delivered to a new mammalian host with the next blood meal.

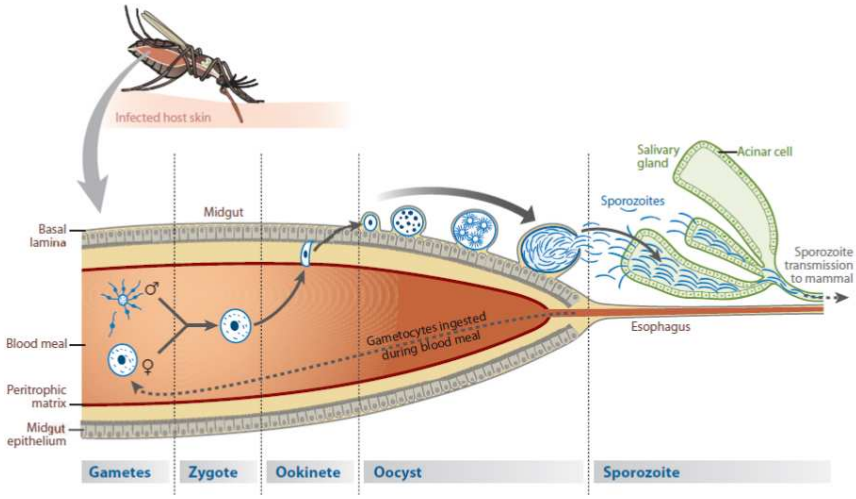

Figure 2.2.3: Mosquito stage (Aly *et al.*, 2009)
When a mosquito takes blood from an infected host, gametocytes end up in the mosquito's midgut, where the sexual reproduction occurs: Exflagellation of the male microgametocyte is followed by the fusion of one male and female gamete, forming a zygote. The zygote matures to a motile ookinete, crosses the epithelium of the midgut and transforms into an oocyst. Sporozoites are formed and released, when the oocyst bursts. They move cranial and cross the acinar cell layer of the salivary glands, from where transmission occurs with the next blood meal.

2.2.3 Liver stage

The infectious form of the *Plasmodium* parasite that is inoculated into the mammalian host is called the sporozoite; a small crescent-shaped cell located in the salivary glands of infected female *Anopheles* mosquitoes. Malaria infection is initiated when sporozoites are delivered into the dermis of the mammalian host through a bite of an infected mosquito (Vanderberg and Frevert, 2004). Most of them leave the dermis by invading a blood vessel (Amino et al., 2006) and reach the liver

sinusoids. Once the sporozoites cross the layer of epithelial and Kupffer cells, they migrate through several hepatocytes until they invade and infect one final hepatocyte (Frevert et al., 2005). The latter occurs by invagination of the host cell membrane (Graewe et al., 2012); thus inside the host cell, the parasite lives within a PV, similar to during the blood stage. The host cell-derived membrane surrounding it, is called the parasitophorous vacuole membrane (PVM) (Mota et al., 2001) and is modified by the parasite by inserting its own proteins (van de Sand et al., 2005). As soon as the parasite has finished the invasion process, it settles close to the host cell nucleus, associating with its endoplasmic reticulum (ER) (Bano et al., 2007), rounds up and develops into a trophozoite (Graewe et al., 2012). The subsequent asexual reproduction step is enormous, resulting in approximately 30,000 nuclei within 35 hours (Baer et al., 2007; Bano et al., 2007). The organelles apicoplast and mitochondrion first elongate and develop into extensively branched networks before being divided into thousands of small apicoplasts and mitochondria (Stanway et al., 2011). In the late liver stage, when parasites have developed into a multinucleated schizont, merozoite formation starts with the cytomere stage: The parasite membrane (PM) invaginates and forms several subunits, where the nuclei are located near the PM (Graewe et al., 2012). The parasite has to manage a perfect segregation organelles into every daughter merozoite. Each divided apicoplast segment finds its place between one nucleus and the membrane, whereas the mitochondrion first forms radial branches towards the nuclei (Stanway et al., 2011; Graewe et al., 2012). When the mitochondrial network is cleaved and the plasma membrane encloses one of each organelle, merozoite formation is completed. Subsequently, the PVM ruptures and thousands of merozoites are liberated into the host cell cytoplasm (Sturm et al., 2006). Simultaneously an unusual but ordered form of host cell death occurs (Heussler et al., 2010). It is speculated to be an autophagy-like programmed cell death (Eickel et al., 2013). Merozoites are delive-

red from the liver tissue to the circulatory system within small membrane-enclosed packets called merosomes, which pinch-off the host cell and burst in the capillaries of the lungs (Sturm et al., 2006; Baer et al., 2007). *In vitro*, merozoite-filled host cells detach from the plastic or glass surface and float in the supernatant, with merosomes budding off these floating cells (Rankin et al., 2010).

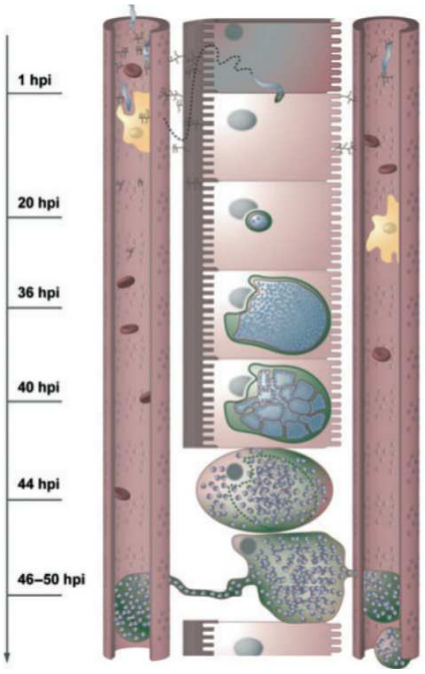

Figure 2.2.4: Liver stage of *P. berghei* (Graewe *et al.*, 2012)
1 hpi: After traversing several hepatocytes, the parasite invades one final hepatocyte, remodels its PVM and becomes a small round trophozoite during the next 24 hpi. 20 hpi: Nuclear division starts. 36 hpi: A huge syncytium, the multinucleated schizont, is formed and contains approximately 30,000 nuclei. 40 hpi: Cytomere stage: The plasma membrane invaginates and forms several units. 44 hpi: The breakdown of the PVM releases the merozoites into the host cell cytoplasm. 46-50 hpi: Merosomes bud off the host cell and get liberated into the blood stream.

Developing intracellularly demands certain survival strategies:

Firstly, the parasite needs the host cell to be prevented from undergoing programmed cell death. In *in vivo* experiments from van de Sand *et al.,* infected hepatocytes did not undergo apoptosis when treated with TNF-α, a known stimulator of liver apoptosis (Wielockx et al., 2001), whereas most uninfected cells

presented degraded DNA. Indeed, *Plasmodium* parasites interact with the host cell and suppress apoptosis (van de Sand et al., 2005). On the molecular level, it has been shown, that the protein "*P. berghei* inhibitor of cysteine proteases" (*Pb*ICP) is involved in this process and is secreted into the host cell during later liver stages *(Rennenberg et al., 2010)*.

Secondly, the parasite needs to establish successful nutrient intake and elimination of waste products. Parasite organelles proliferate extensively and the PV has to grow simultaneously. Thus the parasite needs nucleic acids, amino acids and many membrane elements such as lipids. Interestingly, the parasite associates with the host cell ER (Bano et al., 2007), indicating a role in nutrient or membrane supply. This is maintained by the fact that the liver stage-specific protein 2 (LISP2), a protein exported from the parasite to the hepatocyte during liver stage development, is also detectable within the host cell nucleus (Orito et al., 2013). In addition, the host cell-derived PVM is modified by the insertion of the proteins UIS4 and "exported protein 1" (Exp1). UIS4 does not only localise to the PVM but to a membranous network within the hepatocyte cytoplasm and is speculated to play a role in interactions with the hepatocyte (Mueller et al., 2005). The exact function of Exp1 is not yet elucidated; it is speculated to be involved in the elimination of toxic waste products (Lisewski et al., 2014). For nutrient uptake, there might be open channels in the PVM to transport molecules smaller than 855 Da (Bano et al., 2007; Sturm et al., 2009); larger molecules instead most likely require an active import, for example via endocytosis.

Thirdly, the parasite needs to exit the host cell and prepare the entry of the next host cell. In the *Plasmodium* liver stage, this means the exit of a hepatocyte and entry of a red blood cell, once merozoite formation is completed. The liver stage-specific protein 1 (LISP1) localises to the PVM and is involved in its rupture (Ishino et al., 2009). MSP1, responsible for binding to red blood cells, localises

to the PM already during the schizont stage (Sturm et al., 2009). Later, the PM surrounds the merozoites presenting MSP1 on their surface, which allows them to attach to red blood cells (Herrera et al., 1993; Kauth et al., 2003).

All three capabilities imply an important role of the secretion machinery and intracellular protein transport of *Plasmodium* parasites.

Plasmodium berghei, a rodent malaria model system

P. falciparum is the most virulent species of *Plasmodium* and thus a species of great interest. Liver stage research is, however, difficult to implement: In contrast to merozoites, sporozoites cannot be cultured continuously. Infected *Anopheles* mosquitoes must be dissected to obtain *P. falciparum* sporozoites, thus an S3 insectary would be required. Therefore much research use species, which affect rodents. The rodent malaria model *P. berghei*, first described by Vincke and Lips in 1948 (Vincke and Lips, 1948), is used in this study.

2.3 Intracellular protein transport in eukaryotes

Protein transport is an important element for every eukaryotic cell: It can be used for nutrient uptake via endocytosis, for secretion of proteins via secretory pathway or for communication amongst organelles. In eukaryotic cells, protein transport occurs primarily in or on vesicles, the so-called coated vesicles (CVs). They differ in their coat proteins as well as in their cargo and destinations but are all similar in their architecture; they are composed of an outer layer of coat proteins, an inner layer of adaptor proteins and guanosine triphosphatases (GTPases) and the cargo protein within the curved membrane (Pucadyil and Schmid, 2009).

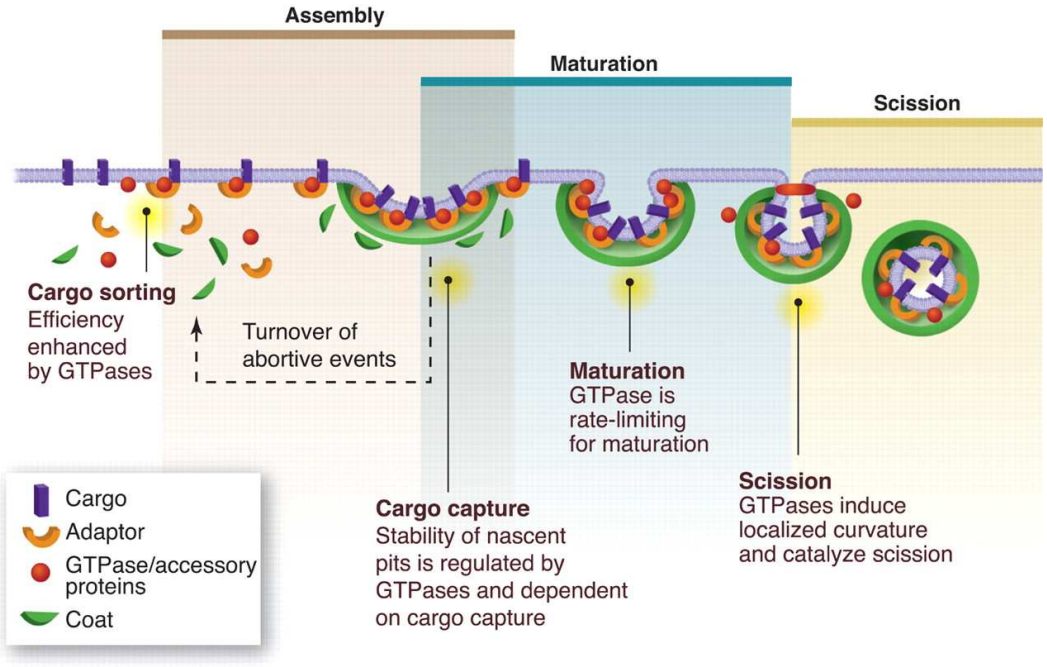

Figure 2.3.1: Coated vesicle formation (Pucadyil *et al.*, 2009)
The three stages of coated vesicle formation: 1. Assembly: Coat subunits accumulate on the membrane, cargo is recognized by the adaptor molecules and coat proteins are recruited. 2. Maturation: Coat protein complexes tend to self-assemble into spherical cages, thus inducing the curvature of the underlying membrane. 3. Scission: CVs are released.

Secretory pathway

In brief, proteins are synthesized within the ER, transported to the Golgi complex, where further protein sorting and modification occurs. From there, they are transported to the plasma membrane and finally released from vesicles by exocytosis (Lee et al., 2004; Schekman and Orci, 1996; Schmid, 1997).

The initial step of the secretory pathway is the transport of proteins from the ER to the Golgi complex, mediated by Coat protein complex II-coated vesicles (COPII CVs). The coat consists of two layers: an outer layer composed of the Sec13-31 protein complex and an inner adaptor layer composed of the small GTPase Sar1 and the Sec23-24 protein complex (Bi et al., 2002). Sar1 is a small GTPase from the Ras superfamily. The protein itself displays only low rates of guano-

sine diphosphate (GDP) dissociation and guanosine triphosphate (GTP) binding and thus requires extrinsic GTP exchange factors (GEFs). The GEF for Sar1 is Sec12, an integral ER-localised membrane protein (Barlowe and Schekman, 1993). Once Sec12 causes the exchange of GDP to GTP, Sar1 forms a membrane-binding site and localises to the membrane of the ER. Formation of the COPII coated vesicle is initiated when Sar1 recruits the adaptor complex Sec23-24 to the membrane (Spang, 2008). This adaptor complex joins the coat complexes Sec13-31 to the cargo molecules. Coat complex proteins characteristically interconnect in a spherical manner and therefore concentrate adaptors and cargo and induce the curvature of the membrane. For the scission of the coated vesicle, hydrolysis of GTP is required. As Sar1 has only a low rate of intrinsic GTP hydrolysis, it needs extrinsic GTPase activating proteins (GAPs). Sec23 is not only an adaptor protein but functions simultaneously as the GAP of Sar1, causing GTP hydrolysis (Bi et al., 2002). Once the COPII CV is cut off, it can make its way to the cis-site of the Golgi apparatus.

Protein transport in *Plasmodium* liver stage

Living inside a host cell calls for the capability of interacting with it. Intracellular parasites have to modify their host cells to ensure their nutrient supply as well as the prevention of any kind of host cell death. This is realised at least in part by trafficking parasite proteins to the host cell, leading to well established secretory systems (Ravindran and Boothroyd, 2008). The parasite-host interaction during liver stage is one aim of current research, and it could be shown that the aforementioned proteins Exp1, UIS4, *Pb*ICP, LISP1 and LISP2 are involved (see 2.2.3). Interestingly, the transport of LISP2 occurs in secretory vesicles surrounded by LISP1 (Orito et al., 2013). Homologues of the aformentioned Sar1 GTPase and Sec31 also exist in *Plasmodium* and indeed are involved in protein transport at least during blood stage (Adisa et al., 2001).

2.4 Aims of this study

The aim of this project was to interfere with the protein transport apparatus of the parasite during liver stage development. As the first part within the secretory pathway, the COPII coated vesicle-mediated transport from the ER to the Golgi seemed a promising target. Takeuchi *et al.* showed that expression of a dominant negative mutant of the Sar1 GTPase of the small flowering plant *Arabidopsis thaliana*, leads to the inhibition of the transport of COPII coated vesicles from the ER to the Golgi (Takeuchi et al., 2000). As there is already evidence for the effective for interference with this protein using a dominant negative mutant approach, Sar1 was chosen for further analysis.

The first goal was to establish transgenic parasites of *P. berghei*, expressing either a wildtype or a mutant form of Sar1 GTPase under the control of the liver stage specific promoter lisp2. Then, the effect of this mutation on liver stage development was tested: parasites expressing either the wildtype or a mutant form of Sar1 GTPase were compared. Additionally, the development was compared to wildtype parasites.

3 Materials and methods

3.1 Materials

3.1.1 Technical and mechanical devices

Table 3.1: Technical and mechanical devices

Type	Model	Manufacture/Distributor
Binocular	KL 1500LCD	Zeiss, Hamburg, D
Cell culture incubators	B5060EK-CO2, B6200	Heraeus, Hannover, D
Centrifuges	4K10 5415C, 5415D, 5810R J2-H5, J2-21	Sigma, Steinheim, D Eppendorf, Hamburg, D Beckmann, Krefeld, D
Confocal microscope	FluoView 1000	Olympus, Hamburg, D
Electrophoresis chamber	Perfect Blue^{TM}	Peqlab, Erlangen, D
Fluorescence microscopes	Axiovert 200 Leitz DM RB SMZ 800	Zeiss, Hamburg, D Leica, Bentheim, D Nikon, Düsseldorf, D
Light microscopes	Axiovert 25, Stremi DV4	Zeiss, Jena, D
Magnetic stirrer	MR Hei-Standard Heidolph	Schwabach, D

Type	Model	Manufacture/Distributor
Microwave	R-208	Sharp Electronics, Hamburg, D
Mosquito incubator	Climacell-707	MMM-Group, Gräfelfing, D
PCR machines	MJ Mini™ Primus 25	Bio-Rad, München, D PEQLAB, Erlangen, D
pH meter	pH Level 1	InoLab, Weilheim, D
Photometer	Bio-Photometer	Eppendorf, Hamburg, D
Pipets	P10, P20, P200, P1000 Research 10	Gilson, Middleton, USA Eppendorf, Hamburg, D
Pipetting aid	Pipetus®-akku	Hirschmann, Eberstadt, D
Power supplies	Consort E835, EV231 EPS 3500	Consort, Turnhout, B Amersham Biosciences, Freiburg, D
Shaker	Innova™4400	New Brunswick, Nürtingen, D
Spectrophotometer	NanoDrop 2000	Peqlab, Erlangen, D
Sterile benches	BSB 6A Herasafe	Gelaire Flow Laboratories, Opera, I Heraeus Instruments, Osterode, D
Thermo incubators	Thermomixer 5436, compact	Eppendorf, Hamburg, D
Transfection device	Nucleofector II	Amaxa biosystems, Köln, D
Tweezers	neoLab-Dumont, Inox Nr. 5	neoLab Migge Laborbedarf, Heidelberg, D
UV transilluminator	UV-Flächenstrahler	Konrad Bender, Wiesloch, D

Type	Model	Manufacture/Distributor
Vortex	VF2	IKA Labortechnik, Hamburg, D
Water bath	Haake Fisons DC1 SW 20	NCM GmbH, Hamburg, D Julabo, Seelbach, D

3.1.2 Labware and disposables

Table 3.2: Labware and disposables

Typ	Specification	Manufacture/Distributor
Cell culture flasks	250 ml	Sarstedt, Nürnbrecht-Rommelsdorf, D
Cell culture plates	24-well	Greiner, Solingen-Wald, D
Coverslips	round 13 mm	Menzel&Glaser, Braunschweig, D
Cuvettes	Halbmikro-Einmal-Küvette 1.5-3 ml	Eppendorf, Hamburg, D
Falcon tubes	15 ml, 50 ml	Sarstedt, Nürnbrecht-Rommelsdorf, D
Glass bottom dishes	Willco®Dish	Willco Wells BV, Amsterdam, NL
Microscope slides	Polysine slides microscope slides SuperFrost®	Mentel GmbH, Braunschweig, D Roth, Karlsruhe, D
Needles	Sterican	Braun, Melsungen, D

Typ	Specification	Manufacture/Distributor
Parafilm	Parafilm "M"®	American Can Company, Greenwich, UK
Pasteur pipets	Pasteur pipet	Brand, Wertheim, D
Petri dishes	9 cm	Sarstedt, Nürnbrecht-Rommelsdorf, D
Plastic pipet tips	10 µl, 200 µl, 1,000 µl	Sarstedt, Nürnbrecht-Rommelsdorf, D
Plastic pipets	5 ml, 10 ml	Sarstedt, Nürnbrecht-Rommelsdorf, D
Reaction tubes	1.5 ml, 2 ml 1.5 ml Nerbe PCR Softtubes 0.2 ml	Eppendorf, Hamburg, D Nerbe plus, Winsen/Luhe, D Biozym, Oldendorf, D
Surgical scissors	13 cm	Hauptner, Zürich, CH
Syringes	BD-Micro-Fine 0.5 ml	BD Medical, Franclin Lakes, USA

3.1.3 Chemical and biological reagents

Table 3.3: Chemical and biological reagents

Chemical or biological reagent	Manufacturer/Distributor
Accutase	PAA Laboratories GmbH, Pasching, A
Agarose	Bio&Sell, Nürnberg, D
Alsever's solution	Sigma, Steinheim, D
Amphotericin B	PAA Laboratories GmbH, Pasching, A
Ampicillin (100 µg/ml)	Sigma-Aldrich, Taufkirchen, D

Chemical or biological reagent	Manufacturer/Distributor
Antibodies	see chapter 3.2.1
Chloroform	Merck, Darmstadt, D
Dako Fluorescent Mounting Medium	Dako, Cambridgeshire, UK
dATP	Roth, Karlsruhe, D
dNTP-Mix	Roth, Karlsruhe, D
EDTA sodium salt	Biomol, Hamburg, D
Ethidium bromide 1%	Biomol, Hamburg, D
Foetal Bovine/Calf Serum (FBS/FCS), 10 %	PAA Laboratories GmbH, Pasching, A
Glycerol	Roth, Karlsruhe, D
Hoechst 33342 (BisBenzimide H33342 trihydrochloride)	Molecular Probes, Leiden, NL
Hyperladder DNA marker I	Bioline, Luckenwalde, D
IPTG (1 mM)	Bioline, Luckenwalde, D
L-Glutamine (100x)	PAA Laboratories GmbH, Pasching, A
LB-Medium (Lennox)	Roth, Karlsruhe, D
Magnesium chloride	Roth, Karlsruhe, D
MEM with Earle's salts without L-glutamine	PAA Laboratories GmbH, Pasching, A
Methanol	Roth, Karlsruhe, D
PBS 1x (phosphate buffered saline)	Invitrogen^{TM}, Karlsruhe, D
Penicillin (100U/ml)/ Streptomycin (100µg/ml)-Mix (100x)	PAA Laboratories GmbH, Pasching, A
PFA 4 % (paraformaldehyde)	Fluka, Steinheim, D
Phenylhydrazine (6 mg/ml)	Sigma, Steinheim, D
Pyrimethamine	Sigma, Steinheim, D

Chemical or biological reagent	Manufacturer/Distributor
Sodium acetate	Biomol, Hamburg, D
Tris	Biomol, Hamburg, D
Wright's staining solution	Sigma, Steinheim, D
X-Gal	Bioline, Luckenwalde, D

Table 3.4: Enzymes and their buffers

Enzyme/Buffer name	Company
Phusion^{TM}High Fidelity DNA Polymerase	New England Biolabs, Frankfurt, D
5x HF-Buffer	Promega, Mannheim, D
Taq Polymerase (GoTaq)	Promega, Mannheim, D
5x GoTaq® Reaction Buffer	Promega, Mannheim, D
T4 DNA Ligase	New England Biolabs, Frankfurt, D
2x rapid ligation buffer	New England Biolabs, Frankfurt, D
BSA, Buffer III and Buffer IV	New England Biolabs, Frankfurt, D
Restriction enzymes: *BamH*I, *Avr*II, *Not*I, *Xba*I, *Apa*I	New England Biolabs, Frankfurt, D

Table 3.5: Stock solutions

Medium	Recipe
Freezing solution for blood stabilates	Alsever's solution with 10% glycerol → stored at -20°C

Medium	Recipe
HepG2 culture medium: "Complete MEM"	MEM with Earle's salts without L-Glutamine 10 % FCS 2 mM L-Glutamine 100 U/ml Penicillin 0.1 mg/ml Streptomycin → stored at 4°C
Infection medium	HepG2 culture medium mixed with 1% of Amphotericin 250 µg/ml final concentration: 2.5 µg/ml Amphotericin → stored at 4°C for a maximum of 4 days
LB-Amp-Medium 1:1000	50 µl Ampicillin 50 ml LB-Medium
LB-Amp-Plates	LB-Amp-Medium with 15 g/l Bacto agar; → autoclave, cool down at 60°C, pour into petri dishes
PBS 10x	100 mM Na_2HPO_4 100 mM NaH_2PO_4 1.5 M NaCl pH 7.4
10x TAE buffer	0.4 M Tris, 0.2 M sodium acetate, 0.01 M EDTA sodium salt, pH 8.0; → stored at room temperature

3.1.4 Kits

Table 3.6: Kits

Kit name	Company
Amaxa® Human T Cell Nucleofector® kit	Lonza, Köln, D
NucleoBond®PC100	Macherey&Nagel, Düren, D
NucleoSpin®Extract II	Macherey&Nagel, Düren, D
NucleoSpin®Plasmid	Macherey&Nagel, Düren, D
QuikChange™Mutagenesis Kit	Stratagene, Frankfurt, D

3.1.5 Software programs and Web Pages

Table 3.7: Software programs

Program	Version	Application
GraphPadPrism	1.0	graphs, statistics
MacVector™	7.2.3	DNA sequence alignments, (plasmid maps)
OpenLab	5.0.2	image acquisition and processing density slicing for measuring parasite size
OpenOffice	3.2	Calc: calculation Draw: image editing
Photoshop®	CS, 8.0	cropping of images, image enhancement, overlays

Table 3.8: Web Pages

Web Page	URL	Application
Plasmo DB	http://plasmodb.org/plasmo/	Plasmodium genomic database
Clustal	http://www.clustal.org/	Alignment of DNA and amino acid sequences

3.1.6 Bacterial strains, cells and parasites

Table 3.9: Bacterial strains, cells and parasites

Name	Type	Purpose
E.coli XL1-Blue	Bacteria	Amplification of plasmids
HepG2 cells	Human hepatoma cell line	Host cell line for *in vitro* experiments with *P. berghei*
P.berghei ANKA	*Plasmodium berghei* laboratory strain	Experiments with *P. berghei*, background strain for transgenic parasites
NMRI mice	Mice	to keep up the whole life cycle of *P. berghei*
Anopheles stephensi	Mosquitoes	to keep up the whole life cycle of *P. berghei*

3.2 Constructs

3.2.1 Antibodies and other staining solutions

Table 3.10: Primary and secondary antibodies

Name	Species	Dilution	Company
anti-ExpI	chicken	1:1000	BNI AG Heussler
anti-V5	mouse	1:2000	Invitrogen, Karlsruhe
anti-GFP	mouse	1:1000	Invitrogen, Karlsruhe
anti-chicken Cy2	goat	1:2000	Dianova, Hamburg
anti-chicken Cy5	goat	1:4000	Dianova, Hamburg
anti-mouse Alexa Fluor® 594	goat	1:6000	Invitrogen, Karlsruhe
anti-rabbit Cy2	goat	1:250	Dianova, Hamburg
DAPI (4'6-diamidino-2-phenylinidol)	-	1:100	Sigma-Aldrich, Taufkirchen

3.2.2 Sar1GTPase with different restriction sites

The whole genomic DNA of Plasmodium berghei is mapped. The corresponding gene of the protein Sar1GTPase is called PBANKA_071880. Its size in gDNA is 1001 bp, the corresponding cDNA is 576 bp because of 2 introns.

Table 3.11: Fragments

internal no.	gene	primers	restriction sites	description
57	wildtype PBANKA_071880	1469, 1470	BamHI	for ligation into pGEM
57*	mutated PBANKA_071880	1469, 1470	BamHI	after mutation of pGEM-57
64	wildtype PBANKA_071880	1511, 1514	NotI, AvrII	for ligation into 17.1.7
65	mutated PBANKA_071880	1511, 1514	NotI, AvrII	for ligation into 17.1.7

3.2.3 Primers

Primers were designed to selectively anneal to the 5' and 3' ends of the gene. Ideally, both primers forward and reverse should have a similar melting temperature, above 60°C and the content of guanine and cytosine (GC) should be close to 50 %. This was not always possible because of the high prevalence of adenine and thymine (AT) in *Plasmodium* genomic DNA Weber (1987). In the primers, restriction sites were included to allow the ligation into plasmids. All primers were purchased from Eurofins Genomics, Germany.

Table 3.12: Primers for gene PBANKA_071880
Restriction sites are underlined, the mutated codon is labeled in red.

Internal No.	Name	Sequence 5'→3'	Restriction enzyme	Discription
1469	RRS0267_1F	ATATGGATCCATGTTTATCATAAATTGGTGAGTTAAATTATAATG	*BamH*I	PCR: "57" of gDNA Sequencing: "57" and "57*"
1470	RRS0267_2R	GCGCGGATCCTGTTAAAAATTGAGATATCCATTTAAAGGC	*BamH*I	
1498	RRS0267_3F	GATTTAAAACATTTGATTTGGGAGGTCTAGAAACAGCAAGAAGGATATGGAG	*Xba*I	QuikChange™ Mutagenesis PCR
1499	RRS0267_4R	CTCCATATCCTTCTTGCTGTTTCTAGACCTCCCAAATCAAATGTTTTAAATC	*Xba*I	
1511	RRS0267_5F	ATATGCGGCCGCATGTTTATCATAAATTGGTGAGTTAAATTATAATG	*Not*I	PCR: "64" and "65" of pGEM Sequencing: "64" and "65"
1514	RRS0267_6R	GCGCCCTAGGCCTGTTAAAAATTGAGATATCCATTTAAAGGC	*Avr*II	

3.2.4 Vectors

Table 3.13: Plasmids

Plasmid	Complete name and Company	Description
pGEM	pGEM-T® Easy Vector System, Promega, Mannheim, D	Cloning plasmid with 3´-T overhangs at the insertion site; has a multiple cloning region within the coding region of the enzyme β-galactosidase allows a blue/white selection.
pGEM-57	-	pGEM after ligation with the fragment 57 (see 3.11)
pGEM-57*	-	pGEM-57 after performing the mutagenesis reaction (see 3.11, 3.4.2)
pL0017	pL0017 MR4, Manassas, USA	Transfection plasmid with a constitutive expression of GFP: EF1α-GFP-3'UTR
pL0017.1.7	modified plasmid pL0017 by A. Nagel	lisp2-(restriction site *Not*I/*Avr*II for Insert)-V5-3'UTR-EF1α-GFP-3'UTR

3.2.5 Parasite strain

Table 3.14: Constructs

Parasite strain	Plasmid	Insert	Description
*Pb*Sar1	pL17.1.7-64	LSA4-Sar1GTPase-V5-3'UTR-EF1alpha-GFP-3'UTR	over-expression of Sar1GTPase during liver stage; constitutive expression of GFP
*Pb*Sar1(H74L)	pL17.1.7-65	LSA4-Sar1GTPase*-V5-3'UTR-EF1alpha-GFP-3'UTR	over-expression of mutant Sar1GTPase during liver stage; constitutive expression of GFP
*Pb*GFP	pL17.1.7	EF1α-GFP-3'UTR	control strain: constitutive expression of GFP

3.3 Protocols

3.3.1 PCR with Phusion™High Fidelity DNA polymerase

In a Polymerase chain reaction (PCR), DNA sequences are amplified exponentially by a heat-stable DNA polymerase in a thermal cycling process. In this project, Phusion polymerase was used for all PCRs.

To start the PCR, an initializing step is necessary to denaturate the DNA (98°C for 30 minutes). Subsequently the thermal cycling starts and is divided into three steps, which were repeated 16-30 times. First the double-stranded DNA chains are denatured and thereby separated (at 98°C). Second the temperature is lowered to 48-55°C allowing annealing of the primers to the single-stranded DNA. Initial reactions, the annealing temperature used was approximately 2°C less than the melting temperature of the primers. Third the enzymatic replication starts with the synthesis of new DNA by addition of dNTPs complementary to the single-stranded DNA template by the polymerase. The elongation temperature depends on the optimum activity temperature of the polymerase (for Phusion 68°C). Elongation time depends on the size of the fragment, approximately 0.5 to 1 minute per 1 kb. After the thermal cycling, a final elongation step is performed to ensure that all single-stranded DNA is fully extended.

Table 3.15: PCR with Phusion™High Fidelity DNA Polymerase:
1. PCR: to amplify the coding sequence of the Sar1 GTPase
2. PCR: first mutagenesis reaction to generate a single stranded plasmid containing a mutated coding sequence of Sar1 GTPase
3. PCR: first mutagenesis reactions to generate a single stranded plasmid with the complementary strain
4. PCR: to amplify the coding sequence of Sar1 GTPase with different restrictions sites
5. PCR: to amplify the coding sequence of the mutated Sar1 GTPase with different restrictions sites

PCR	1. Sar1 of gDNA	2. mutagenesis, vol.1	3. mutagenesis, vol.2	4.	5.
DNA template	1 µl gDNA (= 25 ng)	1 µl pGEM-57 (=10 ng)	1 µl pGEM-57 (=10 ng)	3 µl pGEM-57 (=10 ng)	7 µl pGEM-57* (=10 ng)
Phusion Polymerase 2 U/µl	0.5 µl	1 µl	1 µl	0.5 µl	0.5 µl
HF-buffer 5x	10 µl	10 µl	10 µl	10 µl	10 µl
dNTPs 10 mM	2 µl	1 µl	1 µl	2 µl	2 µl
sense primer (10 pmol/µl)	2 µl 1469	2 µl 1498	-	2 µl 1511	2 µl 1511
anti-sense primer (10 pmol/µl)	2 µl 1470	-	2 µl 1499	2 µl 1514	2 µl 1514
$MgCl_2$1.5 mM	3 µl	2 µl	2 µl	3 µl	3 µl
dH_2O	29.5 µl	33 µl	33 µl	27.5 µl	23.5 µl
total volume	50 µl	50 µl	50 µl	50 µl	50 µl
step					
1. initialisation	98°C, 30 min	98°C, 30 min		98°C, 30 s	
2. denaturation	98°C, 10 s	98°C, 30 s		98°C, 10 s	
3. annealing	48°C, 45 s	55°C, 60 s		48°C, 45 s	
4. elongation	68°C, 45 s	68°C, 4 min		68°C, 45 s	
5. final elongation	68°C, 5 min	4°C, over night		68°C, 10 s	
cycles 2.-4. step	30x	16x		30x	
PCR product	fragment 57	single stranded pGEM-57*		fragment 64	fragment 65

3.3.2 Analysis by gel electrophoresis

DNA is negatively charged and therefore can move in an electric field. Agarose can be used as a matrix in a gel. The speed of the movement depends only on the size of the fragment, smaller molecules travel faster than larger ones. Ethidium bromide added to the sample intercalates into the major grooves of DNA and can be visualised under ultraviolet illumination.

A 1 % agarose gel was made by heating agarose in 1x TAE buffer and mixing it with ethidium bromide (0.1 µg/ml) at a ratio of 1:10,000. The liquid agarose solution was poured into a cast with a comb to create wells for loading the sample. The samples were mixed with loading buffer (here the 5x GoTaq® Reaction Buffer was used as a loading buffer) and loaded on the gel next to 5 µl of the standard Hyperladder I, a marker with DNA fragments in specific sizes (200-10,000 bp) in specific concentrations. This makes it easy to analyse the size and concentration of several DNA fragments, e.g. after PCR or digests. Depending on the size of the gel, voltages between 80 and 115 V were applied for 20-40 minutes in TAE as a running buffer. After this time the gel was viewed with a UV transilluminator with a digital camera.

3.3.3 Purifying DNA

To purify PCR products, the kit NucleoSpin®Extract II was used according to the manufacturer's instructions.

3.3.4 A-tailing reaction

10 µl volume:

4 µl	PCR product
1 µl	GoTAQ
2 µl	GoTAQ-Buffer
2 µl	dATPs 1 mM
1 µl	dH_2O

→ incubation at 70°C for 15-30 min

3.3.5 Ligation

The concentrations of PCR products were estimated by comparison to DNA standards on a gel. To calculate the appropriate amount of insert to include in the ligation reaction, the following equation was used for a 3-fold molar excess of insert DNA:

amount of insert [ng] $= 3x \frac{amount\,of\,vector[ng]}{size\,of\,vector[kb]}\,x\,size\,of\,insert[kb]$

3.3.5.1 Ligation of the coding sequece of *Sar1GTPase* into pGEM®-T Easy Vector

Fragment 57 (see table 3.11) has a size of 1001 bp, the vector pGEM a size of 3 kb. Hence the amount of insert for a ligation with 50 ng vector can be calculated in the following way:

amount of insert [ng] $= 3x \frac{50\,ng}{3\,kb}\,x\,1\,kb = 50$ ng

For the ligation into pGEM reagents supplied with the pGEM®-T Easy Vector System were used.

10 µl volume:

1 µl	50 ng vector
3 µl	A-tailing product
1 µl	T4 DNA Ligase
5 µl	2x L Rapid Ligase buffer

→ incubation at 4°C over night

3.3.5.2 Ligation into the pL0017.1.7 vector

The concentration of PCR products were estimated by comparison to DNA standards on a gel:

concentration of fragment 64: ≈ 30 ng/µl

concentration of fragment 65: ≈ 30 ng/µl

concentration of the vector pL0017.1.7: 50 ng/µl

amount of insert [ng] = $3x \frac{50\,ng}{13\,kb}\,x\,1\,kb = 11.5$ ng ≈ 15 ng

10 µl volume:

1 µl	50 ng vector
0.5 µl	15 ng insert (fragment 64/65)
1 µl	T4 DNA Ligase
1 µl	10x buffer
6.5 µl	dH_2O

→ incubation at room temperature for 1 hour

3.3.6 Transformation

- 5-10 µl Ligation mixed with 50 µl *E. coli* XL1-Blue
- 30 min on ice
- water bath: 60 s, 42°C
- 2 min on ice
- add 950 µl pre-warmed LB medium
- shaker: 37°C, 60 min
- centrifuge: 5,000 g, 5 min
- remove 800 µl Medium
- for ligation into pGEM: add 40 µl 2% X-Gal and 4 µl 0.1 M IPTG)
- resuspend the rest ($\approx$ 200 µl)
- plate on an LB-Amp-plate
- incubate over night at 37°C

3.3.7 Extraction of plasmid DNA

Small-scale extraction:

Clones were picked from LB-Amp plates, inoculated in 3 ml LB-Amp-Medium and incubated in the shaker at 37°C over night. The plasmid contains an Ampicillin resistance gene, which allows bacteria containing the plasmid to multiply. The plasmids were isolated from bacterial cells using the kit NucleoSpin® Plasmid.

Large-scale extraction:

Clones were picked, inoculated and incubated in the same way as described above but incubated during the day. For larger amounts of bacteria and thereby a larger

yield of plasmid DNA, the before inoculated bacteria were added to 200 ml of LB-Amp-medium and incubated over night at 37°C in the shaker. The 200 ml of bacteria were transferred into four 50 ml falcon tubes and pelleted with 5,000 g at 4°C. For further isolation of the plasmid DNA, the kit NucleoBond®PC100 was used.

3.3.8 Digests

After ligation of a specific coding sequence into a plasmid, test digests were carried out to confirm the presence of insert in the plasmid. Another reason for digesting the plasmid was to confirm the presence of an added mutation. This is possible if the mutation leads to an additional restriction site. Preparative digests were performed to open the plasmid and to create compatible ends on the plasmid and insert fragments. Before transfection, the transfection plasmids had to be linearized.

Table 3.16: Digests:
1. test digest of pGEM-57 and pGEM-57* to verify the presence of the insert after ligation; 2. test digest of pGEM-57* to verify the presence of the mutation; 3./4. preparative digest of the fragment 64 and 65 to create compatible ends before ligation; 5./6. preparative digest of the vector 17.1.7 to create compatible ends before ligation; 7. test digest of 17.1.7-64 and -65 to verify the presence of the insert after ligation; 8. preparative digest to open the plasmids 17.1.7-64 64 and 65 before transfection

Number of digest	1	2	3	4	5	6	7	8
template	pGEM-57/-57*	pGEM-57*	fragment 64/65	digest 3	17.1.7	digest 5	17.1.7-64/-65	17.1.7-64/-65
digested with	*BamH*I	*BamH*I/ *Xba*I	*Not*I	*Avr*II	*Not*I	*Avr*II	*Not*I/ *Avr*II	*Apa*I
DNA template	5 µl	5 µl	20 µl	20 µl	4 µl	20 µl	2 µl	8 µl
1. restriction enzyme	1 µl	1 µl	1 µl	-	1 µl	-	0,25 µl	2 µl
2. restriction enzyme	-	1 µl	-	1 µl	-	1 µl	0,5 µl	-
BSA 10x	3 µl	3 µl	4 µl	4 µl	4 µl	4 µl	1 µl	3 µl
Buffer III	3 µl	3 µl	4 µl	-	4 µl	-	1 µl	-
Buffer IV	-	-	-	4 µl	-	4 µl	-	3 µl
dH_2O	18 µl	17 µl	11 µl	11 µl	27 µl	11 µl	5,25 µl	14 µl
volume	30 µl	30 µl	40 µl	40 µl	40 µl	40 µl	10 µl	30 µl
incubation	37°C 45-60 min	37°C 120 min	37°C 90 min	37°C 90 min	37°C 90 min	37°C 90 min	37°C 90 min	room temperature, 30 min 37°C, 90 min

3.3.9 Sequencing

For sequencing, 600-700 ng DNA was required. The DNA concentrations of selected samples were estimated with a Photometer and if necessary diluted with distilled water. The samples were mixed with the according forward and reverse primers whose sequences lie either side of the insertion site (same primers as used for the PCR, see chapter 3.2.3). The mixture was sent to SeqLab (Sequence Laboratories Göttingen GmbH, Göttingen, D) for sequencing.

3.4 Creating genetically modified parasites

To create genetically modified parasites, Janse *et al* Janse et al. (2006) developed a method to transfect schizont stage parasites with linearised plasmids in a simple procedure (see chapter 3.3.6). In this work, DNA sequences designated for transfection were ligated into a specific transfection plasmid (pL0017.1.7, see table 3.13): The full-length coding sequence for Sar1 GTPase as well as the mutant version were introduced into the plasmid, resulting in liver stage-specific expression of the proteins with a V5 tag.

Site-specific mutations can be easily induced by a mutagenesis reaction using the the QuikChange™ Mutagenesis Kit (see chapter 3.4.2). This uses a whole plasmid as a template in a PCR reaction. Thus the coding sequence was first cloned into the pGEM-T® Easy Vector System (see chapter 3.4.1) for subsequently performed mutagenesis reactions. The cloning into the final transfection plasmid pL0017.1.7 was carried out afterwards (see chapter 3.4.3).

All plasmids were transformed into the *Escherichia coli* XL1-Blue to generate a convenient and abundant source of plasmid (see chapter 3.3.6).

3.4.1 Cloning into pGEM®-T Easy Vector System

The coding sequence of the Sar1GTPase was amplified with the Phusion™High Fidelity DNA polymerase in a standard PCR reaction (see chapter 3.3.1) resulting in the fragment "57" (see table 3.11).

To verify the correct size of the amplified fragment, 2 µl of the PCR product was loaded on an agarose gel next to 5 µl of the standard Hyperladder I (see chapter 3.3.2). PCR products were purified (see chapter 3.3.3), another control gel was made and showed a bright band at 1001 bp (the size of the amplified gene).

The purified PCR product "fragment 57" was first cloned into the pGEM®-T Easy Vector which has a 5'-T overhangs at the insertion site, which anneal with 3'-A overhangs. The Phusion™High Fidelity DNA polymerase has an additional proofreading activity but generates blunt-end fragments. Thus a seperate A-tailing reaction by Taq polymerase (GoTaq) was necessary (see chapter 3.3.4).

Ligation into pGEM® was made by using the reagents supplied with the pGEM®-T Easy Vector System (see chapter 3.3.5) resulting in the plasmid "pGEM-57" (see 3.13).

To amplify the vector, plasmids were transformed in competent *E. coli* XL1-Blue (see 3.3.6) and plasmid DNA was extracted afterwards (see chapter 3.3.7).

To see if the clones contain the correct insert, a test digest was prepared with the restriction enzyme *BamH*I, which cuts the insert out of the plasmid (see chapter 3.3.8). The digest was loaded on a gel and showed the expected two bands at 3 kb (pGEM) and 1 kb (insert).

Clones with the correct size of the insert were sent to sequencing (see chapter 3.3.9) and showed the correct sequence.

3.4.2 QuikChange™ Mutagenesis

The vector pGEM-57 was used as a template for QuikChange™ Mutagenesis PCR.

Primer design

The desired mutation should change the histidine residue amino acid "74" into a leucine residue. The codon of the two amino acids differs in two base pairs ("CTA" instead of "CAT"). Two oligonucleotide primers, each complementary to opposite strandes of the vector, were designed containing the desired point mutation.

Because of the high content of adenine and thymine in the genome Weber (1987) it was not possible to design primers exactly the way the protocol of the QuikChange™ Mutagenesis Kit suggested. The designed primers are shown in table 3.13.

The mutagenesis reaction is divided into two steps:

The first mutagenesis reaction is a PCR with two primers used separately to generate single stranded plasmids (see chapter 3.3.1). In this specific PCR reaction, the whole plasmid pGEM-57 is used as a template and is amplified. For the second mutagenesis reaction, 25 µl of each of these complementary single stranded plasmids were combined together as well as with an additional 1 µl of the Phusion DNA Polymerase. The thermal cycling was the same as for the first reaction with one difference: Directly after the 16^{th}cycle, 1 µl of the enzyme *Dpn*I (10 U/µl) was added. The *Dpn*I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from *E. coli* strains is dam methylated and therefore susceptible to *Dpn*I digestion. Incubation occured at 37 °C for one hour.

Amplifying pGEM-57*

Transformation, inoculation, selection, purifying and digestion was carried out as described before (see chapters 3.3.6, 3.3.7 and 3.3.8).

Verifying the mutation

The chosen mutation led to a sequence including a restriction site for *Xba*I. Therefore an additional digest with *BamH*I and *Xba*I can be used as a testing tool for the induced mutation: It should lead to two inserts with the approximately size of 650 and 350 bp (see chapter 3.3.8). If no *Xba*I site had been introduced, inserts would have the size of 1001 bp.

Via gel electrophoresis (see chapter 3.3.2), clones containing the mutation were identified, subsequently sequenced by SeqLab (see chapter 3.3.9) and showed the correct mutation.

3.4.3 Cloning into pL0017.1.7

The pL0017 vector obtained through the MR4 (MRA-786) is a *Plasmodium berghei* transfection plasmid and allows constitutive expression of GFP under the control of the ef1α promotor, either by episomal expression or by integration of plasmid sequence into the cssu/dssu ribosomal gene locus of the parasite for insertion (single crossover).

pL0017.1.7 is a variation of the pL0017 vector and was designed by A. Nagel. It contains the liver-specific promotor lisp2 Helm et al. (2010) instead of the constitutive ef1α promotor. It has a *Not*I and a *Avr*II restriction site. Here it is shortly called 17.1.7.

At first a PCR with different primers was performed to amplify the gene with the desired restriction sites *Not*I and *Avr*II (see chapter 3.3.1) resulting in the

fragments 64 and 65 (see table 3.11). Subsequently the PCR product was purified and the correct size was confirmed via electrophoresis. The PCR product as well as the vector 17.1.7 were digested with the two mentioned restriction enzymes (see chapter 3.3.8). The vector 17.1.7 was then ligated with the fragment 64 and 65 (see chapter 3.3.5).

Transformation, inoculation, purification and digestion were done as described before (see chapters 3.3.6, 3.3.7 and 3.3.8). The clones with the correct size of the insert were sent to sequencing (see chapter 3.3.9) and showed the correct sequence.

3.4.4 Transfection

Parasites were transfected as previously described from Janse *et al.* Janse et al. (2006) with some minor changes. The transfection is realized by electroporation of schizont stage parasites with plasmid DNA. It was performed by Rebecca Stanway using the Amaxa® Human T Cell Nucleofector® kit.

The transfection plasmid pL0017 and its modified version pL0017.1.7 contain a coding sequence conferring resistance to pyrimathamine, which allows a selection of successfully transfected parasites. One day after transfection, 7 mg/l of pyrimethamine was added to the mice's drinking water to kill all wildtype parasites. After 7-16 days after transfection, parasites can be detected in a blood smear.

To obtain genetically modified sporozoites, mosquitoes were allowed to take a blood meal from the infected mice (see chapter 3.5).

3.5 Culture of *Plasmodium berghei*

In this project the *P. berghei* ANKA strain was used. This strain was described by Vincke and Lips in 1948 (Vincke and Lips, 1948). For transgenic parasites, the wildtype strain was transfected with a plasmid containing the desired genes.

It is not possible to culture the liver stage of *Plasmodium* continuously (as is possible with the blood stage). However, the rodent model *P. berghei* can be used to study the whole life cycle of the parasite. An NMRI-mouse is infected by intraperitoneal injection of conserved infected blood (see section 3.5). When the parasitemia is between 5 and 15 %, the blood from this mouse is transferred into a second mouse that had been treated with 200 µl Phenylhydrazine (6 mg/ml in PBS) 2-3 days earlier. This treatment kills red blood cells, which leads to an increased production of reticulocytes, the preferred host cells of *P. berghei*. This makes the parasitemia come up very fast and produces many gametocytes, the sexual stage of the parasite. The parasitemia and the correct development of the gametocytes are checked by looking at a blood smear. 3 days after infecting the second mouse (parasitemia: 10-15 %), female mosquitoes of the species *Anopheles stephensi* are fed with this blood and thus become infected themselves. In the mosquito occurs the sexual reproduction of *Plasmodium*. Approximately 18 days later sporozoites can be isolated from the salivary glands of the mosquitoes and used for *in vitro* infection of HepG2 cells.

Determination of parasitemia

Parasitemia was determined by looking at a blood smear from the infected mouse. For this purpose a tiny bit of the mouse's tail was cut off with scissors and 1 drop of blood from the tail vein was put on a microscope slide. Smearing this drop with another slide, a thin blood smear was prepared easily. The dried blood smear was stained with 10 drops of Wright's staining solution, incubated for 2 minutes

and diluted with 10 drops dH_2O for another 2 minutes. Subsequently the slide was rinsed with tap water and air-dried. Under the light microscope, infected and non-infected red blood cells were counted and the parasitemia calculated:

parasitemia [%] = $\frac{infected\,cells}{non\,infected\,cells} * 100$

Blood stabilates

Blood stabilates were performed in aliquots of 100 µl of blood at a parasitemia of 5-15%. Each aliquot was mixed with 200 µl of freezing solution (see table 3.5) and rapidly frozen in liquid nitrogen.

3.6 Culture of *Anopheles stephensi*

Mosquitoes of the genus *Anopheles stephensi* were cultivated in a climate controlled room with 27°C and 80% humidity and a 12-hour/12-hour light-dark cycle. Adult animals were kept in cages and fed with 8 % fructose solution. To induce egg production, female mosquitoes require blood feeding. This was realised by offering human blood in a membrane feeding device once or twice a week.

To infect the mosquitoes with *P. berghei*, approximately 150-200 mosquitoes were kept together in one cage. The blood meal was then performed by putting an infected anaesthetised mouse (parasitemia 10-15%) on the cage.

3.7 Culture and infection of HepG2 cells

HepG2 cells derive from a human hepatocellular carcinoma obtained from European Collection of Cell Cultures (ECCC).

3.7.1 Culture

Cells were cultured in 250 ml cell culture flasks filled with 20 ml culture medium in an incubator at 37°C with 5 % CO_2. Every 3-4 days, cells were washed with PBS before adding 2 ml accutase. Cells were incubated at 37°C for 10 minutes. Fresh culture medium was added and cells were spun down for 5 minutes at 200 g. Supernatant was removed and cells were resuspended in 5 ml medium. 1 ml of this cell suspension was seeded in a new 250 ml cell culture flask.

3.7.2 Infection

Cells were counted with a Neubauer counting chamber and 40-100,000 cells were seeded per well either in a 24-well plate (with/without cover slips) or in glass bottom dishes filled with infection medium.

3.7.2.1 Preparation of Sporozoites

18-26 days after the blood meal (see chapter 3.5), mosquitoes contained sporozoites in their salivary glands.

Mosquitoes were anaesthetised with chloroform, sterilised superficially with ethanol and then dipped in PBS. Under a stereomicroscope with 20-50x magnification, the head of the mosquito was removed from the thorax carefully. The salivary glands were then dissected from either the head or the thorax and placed in infection

medium. After dissecting the salivary glands, they were disrupted mechanically so that sporozoites were released into the medium.

3.7.2.2 Infection of HepG2 cells

HepG2 cells were seeded the day before infecting. To each well or dish, released sporozoites from 2-4 mosquitoes were added. The cells were put in the incubator and sporozoites could invade the hepatoma cells. Medium was changed after 2 hours and then twice a day.

3.7.3 Fixing cells

Cells were seeded on cover slips in 24-well-plates (see chapter 3.7). For fixing at 24 hpi, 60,000 cells were seeded per well. For fixing at later timepoints, only 40,000 cells were seeded per well. The cells of each well were infected with sporozoites derived from 2-4 mosquitoes.

To fix the cells, the cover slips had to be removed carefully from the well and put in a new well filled with 1 ml fresh infection medium. Cells were washed 3 times with 1 ml PBS before being fixed with 0.5 ml of PFA, incubating for 20 minutes at room temperature. Cells were washed again once with 1 ml PBS and subsequently 1 ml of ice cold methanol (-20°C) was added to each well. The plates were surrounded with tape to protect them against drying-out and stored at -20°C in methanol.

3.8 Analysis of transgene parasites during liver stage

3.8.1 Counting infected cells

In a 24-well-plate, 60,000 cells were seeded per well and each infected with sporozoites from 2-4 mosquitoes (see chapter 3.7.2.2). For each parasite strain (*Pb*Sar, *Pb*Sar(H74L) and *Pb*17.1.7) 4 wells were infected at a time.

All 3 transgenic parasites expressed constituvely GFP so that counting the cells was feasible under a basic fluorescence microscope. The counting was carried out by meandering the well at 24, 48, 65 and 72 hpi (see Fig. **??**). At 65 hpi, the parasite has reached late liver stage and usually fills the complete host cell with merozoites. From the subsequently detached cell, merosomes bud off. To compare the number of floating cells to infected cells at 24 and 48 hpi, to assess parasite maturation, it had to be ensured not to count the merosomes but only the number of detached cells. For this purpose, nuclei were stained with Hoechst 33342 (1 µg/ml for 10 minutes at 37°C), which allowed differentiation between merosomes and detached cells.

3.8.2 Sizing parasites

To assess the development of the transgenic parasites, the growth was analysed by measuring their size at different time points.

Conditions: 9 wells with 50,000 cells/well, each infected with sporozoites derived from 3 mosquitoes; 3 wells per parasite strain.

Density slicing:

A minimum of 40 pictures of each parasite strain was taken with a digital camera at 24, 48 and 56 hpi.

For measuring the size of the parasites, the program OpenLab was used with its funcion "density slicing", which calculates the area of each parasite in 2D.

3.8.3 Live imaging

For live imaging, glass bottom dishes were used with 100,000 HepG2 cells per well. For both *Pb*Sar and *Pb*SarH74L, two dishes were infected, each with sporozoites of 3 mosquitoes. Medium was changed after 3 hours and twice a day as described before (see chapter 3.7.2.2).

At 24, 48 and 54 hpi, the cells were stained with Hoechst 33342 (1:20,000) and looked at with fluorescence microscopy. Pictures were taken with a digital camera.

3.8.4 Immunofluorescence assays

Fixed cells (see chapter 3.7.3) were washed with PBS five times before being blocked with 0.5 ml 10 % FCS/PBS and incubated 1 hour at room temperature.

All antibodies were used at specific dilutions (see table 3.10) with 2 % FCS/PBS. The primary antibodies were mixed together and 65 µl were dropped on parafilm. The cells on the coverslip were then placed into the drop. The staining occured for approximately 1 hour at room temperature in a humid chamber. Before proceeding with the secondary antibodies, the cells were washed again with PBS. The procedure for the secondary antibodies was the same as for the primary ones. After staining, cells were again washed with PBS 5 times and then rinsed with dH_2O. The cover slips were put on mounting medium on a microscopy slide and stored at 4°C.

Staining for general expression patterns:

Primary antibodies: anti-Exp1 (chicken), anti-V5 (mouse) and anti-GFP (rabbit).

Secondary antibodies: anti-chicken Cy5, anti-mouse Alexa594, anti-rabbit (Cy2) and DAPI.

With confocal fluorescence microscopy, the localisation of expressed proteins was analysed (see chapter 4.1.3).

Staining for Exp1 trafficking:

Primary antibodies: anti-Exp1 (chicken), anti-GFP (mouse)

Secondary antibodies: anti-chicken (Cy2), anti-mouse Alexa 594

With normal fluorescence microscopy, the PVM of the parasite was analysed (normal/abnormal/no Exp1 staining) (see chapter 4.4).

4 Results

4.1 Generation of Sar1 GTPase and Sar1 GTPase(H74L)-expressing parasites

In brief, the Sar1 GTPase coding sequence was amplified by PCR and ligated into the intermediate pGEM®-T-easy plasmid. Following site-directed mutagenesis to introduce the required mutation into the mutatnt coding sequence, the wild-type or mutant coding sequence were sub-cloned into the plasmid pL0017.1.7, upstream of a V5 coding sequence and transfected into *P. berghei* parasites.

4.1.1 The predicted Sar1 GTPase of *P. berghei* shows strong similarity to that of other organisms

The genome of *P. berghei* encodes a Sar1 GTPase that has high similarity to that of other species, including plants and yeast (see Fig. 4.1.1). In particular, the GTP-binding motif and effector domain are similar to those of other species, so it is likely that the protein is active and can perform the same function as in other systems. The amino acid sequence includes a conserved histidine residue (H74) near the GTP-binding area, whose mutation in other species hinders the activation through its GTPase-activating protein. Sar1 is locked in the GTP-bound state

and thus hinders COPII vesicle formation and subsequently ER to Golgi protein transport (Saito et al., 1998; Takeuchi et al., 2000).

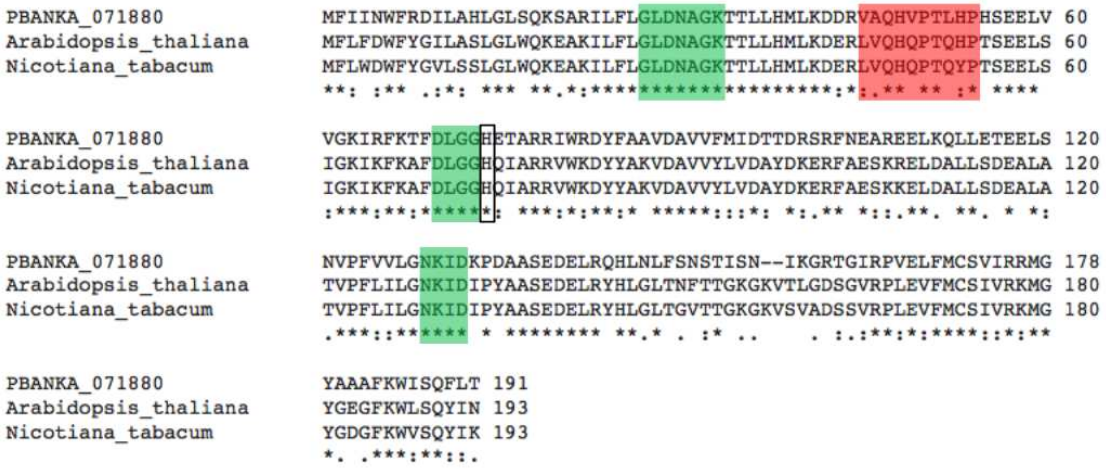

Figure 4.1.1: Alignment of the amino acid sequence of Sar1 GTPase of *Plasmodium berghei*, *Arabidopsis thaliana* and *Nicotiana tabacum*
Green labeling indicates the GTP-binding consensus motif. Red labeling indicates the effector domain. The conserved histidine residue H74 is shown with a black box.
***** = all three species match; **.** = no match between the species; **:** = only two of the three species match

4.1.2 Cloning of plasmids containing the wildtype or mutant coding sequence of Sar1 GTPase

After amplification and ligation of the Sar1 GTPase coding sequence into the intermediate pGEM®-T-easy plasmid, site-directed mutagenesis was performed (to introduce the required mutation) and verified by digesting the plasmid with *BamH*I and *Xba*I (see section 3.4.2). The coding sequence of Sar1 was cut out by *BamH*I, resulting in two products: the plasmid pGEM®-T-easy with approximatly 3,000 bp and the insert with 1,001 bp. The mutated coding sequence were cut equally with an additional cut through the mutation by *Xba*I, resulting in 3 products: the plasmid and two parts of the insert with 350 and 650 pb respectively.

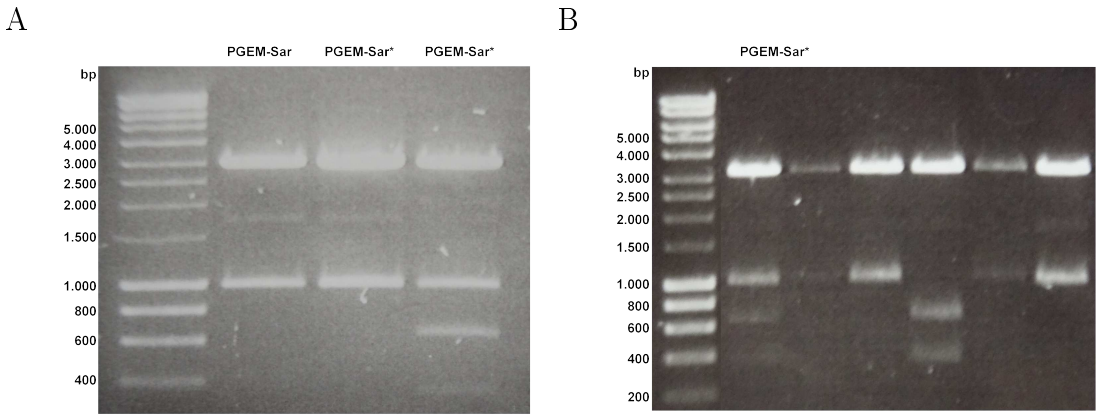

Figure 4.1.2: Gel electrophoresis of the digest of pGEM®-T Easy plasmid containing the coding sequence of Sar1 GTPase.

The restriction enzymes used cut between the insert and the plasmid as well as within the mutation, resulting in 2 (3 and 1 kbp) or 3 products (3 kbp, 650 and 350 bp) respectively.

A: First lane: standard Hyperladder I; second lane: wildtype coding sequence of the Sar1 GTPase; third and fourth lane: after mutagenesis. In the third lane, there are only two products (3 and 1 kbp), implying that mutagenesis was unsuccessful. In the fourth lane, there are four products; an additional band at 1,000 bp is seen, implying that not every insert is cut. Sequencing of the fragment showed a mixture of wildtype and mutant coding sequence.

B: First lane: standard Hyperladder I; other lanes: after retransformation of the mixed coding sequence. In the fifth lane, 3 products with the correct size (350 and 650 bp) can be seen, implying the corresponding plasmid exclusively contains the mutated coding sequence.

The wild-type or mutant coding sequence were sub-cloned into the plasmid pL0017.1.7, a plasmid containing the liver-specific promotor lisp2, followed by 2 restriction sites, the V5-tag as well as the additional cassette of the constitutive GFP expression: lisp2-(restriction site *Not*I/*Avr*II for Insert)-V5-3'UTR-ef1α-GFP-3'UTR (see section 3.2.4). At first, a PCR with suitable primers was performed to extract the coding sequence with the fitting restriction sites.

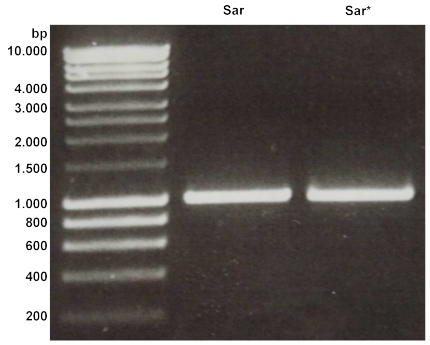

Figure 4.1.3: PCR of the coding sequence of the Sar1 GTPase of pGEM®-T-easy plasmid.
Both, the wildtype and the mutated coding sequence have a length of approximately 1,000 bp. First lane: standard Hyperladder I; second lane: coding sequence of the wildtype Sar1; third lane: coding sequence of the mutated Sar1.

The PCR product was ligated into the plasmid pL0017.1.7, transformed into *E. coli*, inoculated and digested with the restriction enzymes *Not*I and *Avr*II.

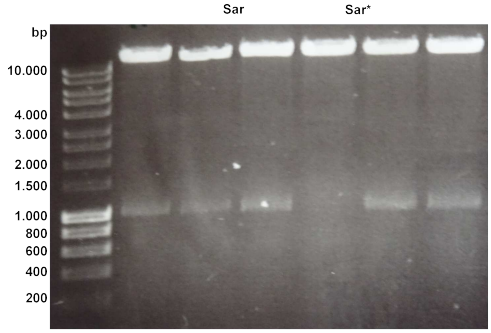

Figure 4.1.4: Test digest of the plasmid pL0017.1.7 ligated with the coding sequence of wildtype or mutated Sar1 GTPase.
First lane: standard Hyperladder I; lane 2-4: pL0017.1.7 ligated with the wildtype sequence of Sar1; lane 5-7: pL0017.1.7 ligated with the mutated sequence of Sar1. In the fourth lane the ligation was not successful.

Successfully cloned plasmids were transfected into *P. berghei* parasites. The two plasmids are called p^{lisp}Sar1-V5$^{ef1\alpha}$GFP and p^{lisp}Sar1(H74L)-V5$^{ef1\alpha}$GFP, respectively (see table 3.13). The corresponding parasites are called *Pb*Sar1 and *Pb*Sar1(H74L). To check that expressing the wild type Sar1 GTPase has no effects on the development itself, parasites transfected with only the pL0017.17 plasmid were used as a control, which are referred to as *Pb*GFP.

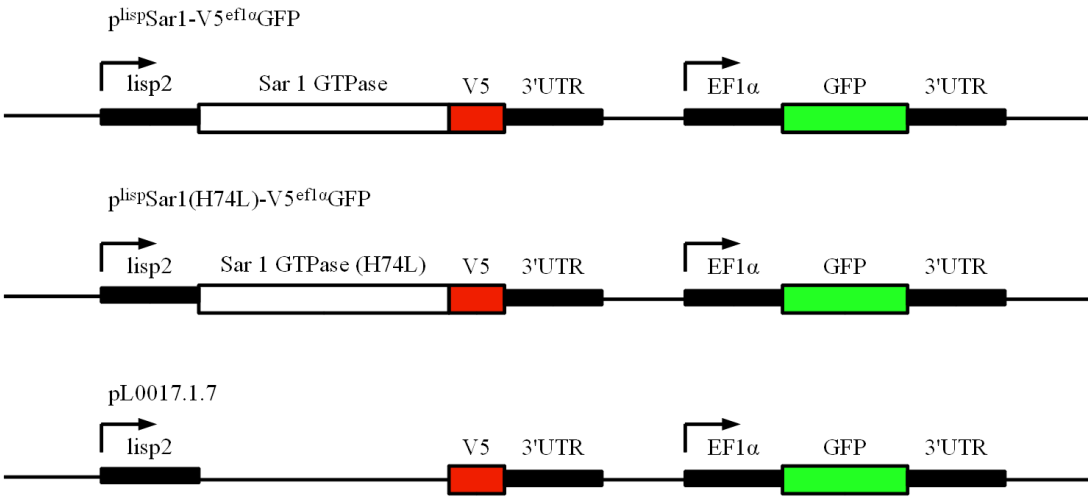

Figure 4.1.5: Plasmid map of p^{lisp}Sar1-V5$^{ef1\alpha}$GFP and p^{lisp}Sar1(H74L)-V5$^{ef1\alpha}$GFP (not to scale); in each case the 3'UTR is the 3'UTR of the *Pb*dhfr/ts

P. berghei parasites were generated, expressing either the wild type or a mutant form of the Sar1 GTPase under the control of the liver stage-specific promoter lisp2, each tagged C-terminally with the V5 peptide (see Fig. 4.1.5). The mutation was a simple point mutation and resulted in a replacement of the histidine at position 74 with a leucine residue. For live imaging experiments and for visualisation of the transgenic parasites in all stages, a second expression cassette was included, resulting in the constitutive expression of cytosolic GFP under the ef1α promotor.

4.1.3 Transgenic parasites indeed express the V5-tagged Sar1 GTPase

Before starting experiments with parasites expressing the dominant negative mutant form of Sar1 GTPase, the expression of the V5-tagged Sar1 GTPase under the control of the liver stage-specific promoter lisp2 was tested. To prove this, only non-mutated parasites were used. This decision was made in particular to reduce the number of mice, implying that a punctual mutation would have no effect on the transcription itself.

Transfected parasites were selected by a treatment with pyrimethamine, to which wildtype parasites have no resistance. Still, reaching the liver stage, many parasites had reverted to wild type, presumably having lost the transfected plasmid. In order to establish whether GFP-expression could be used to indicate which parasites were transgenic and so expressing Sar1 GTPase, immunofluorescence analysis was performed on fixed parasites at 48 hpi, staining for the V5 tag as well as for GFP.

In Fig. 4.1.6, Sar1 GTPase-expressing transgenic parasites are compared to wildtype parasites. Both parasites present broad Exp1-staining, a known molecular marker of the PVM (Sanchez et al., 1994), whereas only the transgenic parasite shows GFP staining. With an anti-V5 antibody it is shown that the transgenic parasite indeed expresses the V5-tagged Sar1 GTPase, implying this is correct for the mutant protein as well. Subsequent analysis was carried out on both non-mutant and mutant GFP-expressing parasites, implying that these would also express the V5-tagged Sar1 GTPase protein.

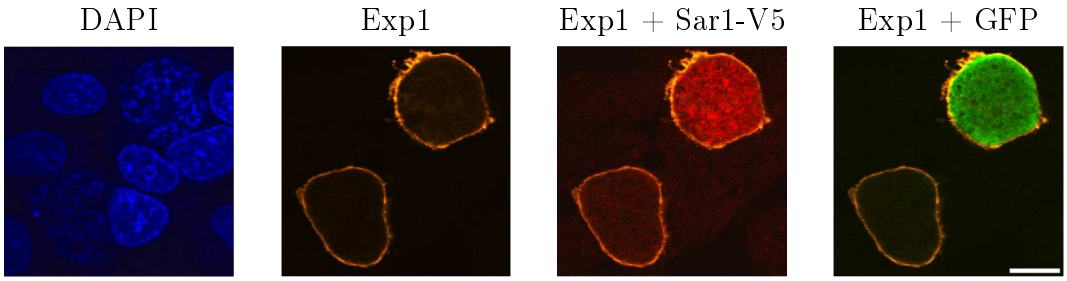

Figure 4.1.6: *P. berghei* transfected with a Sar1 GTPase-V5 construct express the fusion protein during the liver stage.
HepG2 cells were infected with *Pb*Sar1 sporozoites, fixed at 48 hpi and stained with DAPI (blue), anti-Exp1 (orange), anti-V5 (red) and anti-GFP (green). Analysis by confocal microscopy shows that transgenic parasites indeed express the V5-tagged Sar1 GTPase. Anti-V5 antibody stains the Sar1 GTPase localised in the cytoplasm, anti-Exp1 stains the PVM. Note that in the picture, a transfected parasite expressing GFP and the V5-tagged Sar1 GTPase and a wildtype parasite are visible. The latter has presumably lost its plasmid. Scale bar: 10 µm.

4.2 Sar1 GTPase(H74L)-expressing parasites show a defect in liver stage development *in vitro*

As the development of *Pb*Sar1(H74L) parasites was hypothesised to be abnormal, experiments were established to quantify parasite development. During the liver stage, *Plasmodium* parasites expand enormously during their replication period to finally harbor thousands of merozoites. After PVM break down, infected host cells detach from the cell culture dish surface and float in the supernatant. Thus, the size of parasites and their conversion rate to detached cells were chosen as criteria as a measure of a normal development.

*Pb*Sar1(H74L) parasites show a reduced growth

The cytosolic GFP expression of all transgenic parasites allows estimation of the size of the Sar1 GTPase-expressing parasites during liver stage development. In-

fected hepatoma cells were photographed at different time points and parasite area was calculated using a software program (OpenLab®). There was no significant difference in size at 24 hpi between *Pb*Sar1 and *Pb*Sar1(H74L) parasites. However, at the later timepoints of 48 and 56 hpi, the size of the mutant parasites *Pb*Sar1(H74L) was drastically reduced compared to both non-mutant *Pb*Sar1 and control *Pb*GFP parasites (see Fig. 4.2.1). Interestingly, although *Pb*Sar1(H74L) parasites continued growing after 48 hpi, they did not reach the size of normal 48 hpi parasites during course of the 72 hpi period for which size was measured and remained significantly smaller in size.

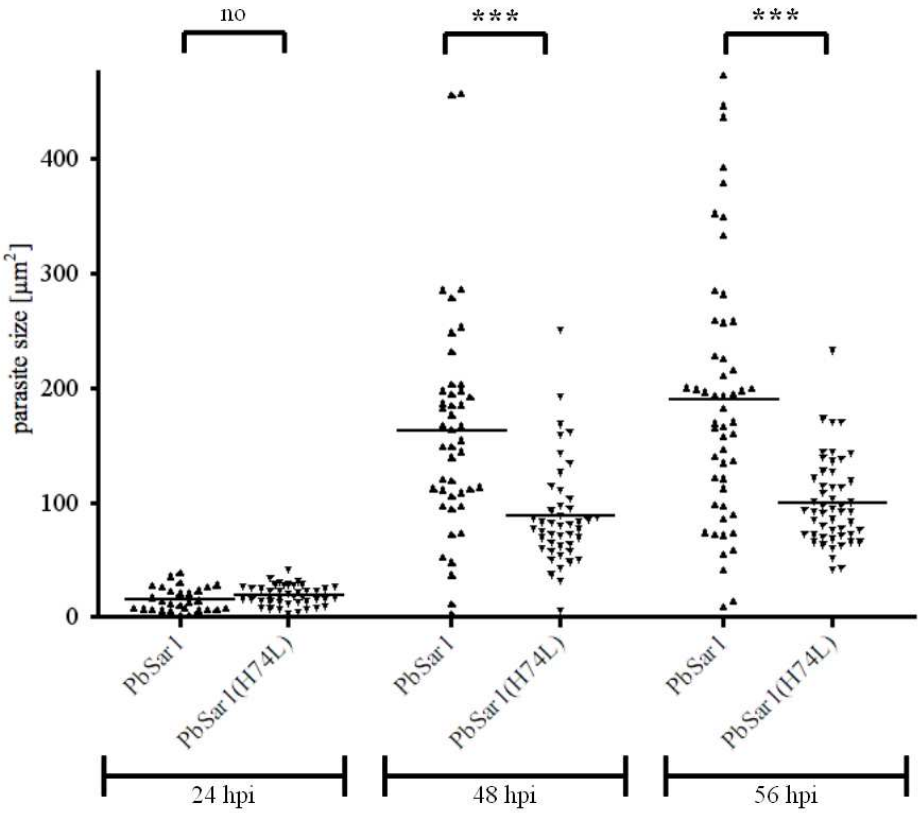

Figure 4.2.1: Comparison of size of *Pb*Sar1 and *Pb*Sar1(H74L) parasites during liver stage development at different time points.
Each spot represents one parasite. In the early liver stage, at 24 hpi, no difference in size can be seen. At the later time points (48 and 56 hpi) *Pb*Sar1(H74L) parasites show drastically reduced growth (*** = $p < 0.05$) compared to *Pb*Sar1 parasites.

Reduced conversion rate

During liver stage development, the parasite grows and forms a multinucleated schizont before developing into thousands of merozoites. Subsequently, the PVM breaks down and releases merozoites into the host cell cytoplasm. *In vitro*, the host cell rounds up, detaches from the plate and floats in the supernatant. The conversion rate is the percentage of detached cells relative to the number of parasites at 48 hpi.

In *Pb*Sar1 parasites, conversion rate showed a wide variation, but no statistically significant difference was seen compared to the control *Pb*GFP parasites. In contrast, *Pb*Sar1(H74L) parasites show a drastically reduced conversion rate of 0.4 %, compared to the average of 24 % seen for the *Pb*Sar1 parasites (see Fig. 4.2.2).

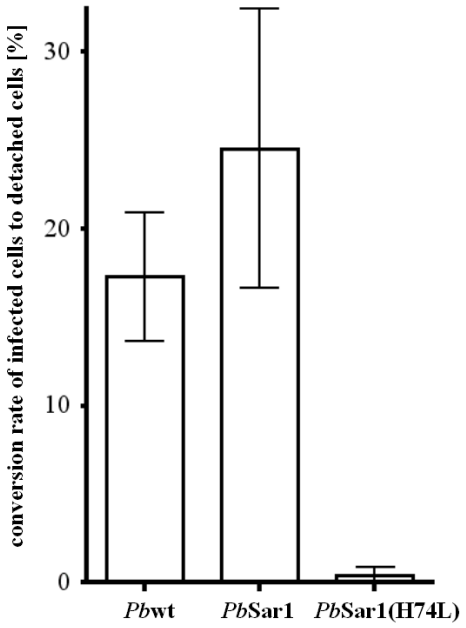

Figure 4.2.2: Conversion rate of infected cells to detached cells
The number of detached cells compared to the number of parasites at 48 hpi drastically reduced in *Pb*Sar1(H74L) parasites ($p < 0.05$). *Pb*Sar1 parasites show no statistically significant difference compared to *Pb*GFP parasites ($p>0.05$).

Both results show a strong influence of protein transport on growth and development during the liver stage. Interfering with the secretory pathway impairs the parasite's ability to form fully developed merozoites.

For the subsequent experiments, only *Pb*Sar1 and *Pb*Sar1(H74L) were used; the control *Pb*GFP was excluded. As the results until now showed no differences between *Pb*Sar1 and *Pb*GFP, it was decided that no longer comparing the two Sar1-expressing strains to *Pb*GFP would allow mice to be saved.

4.3 Sar1 GTPase(H74L)-expressing parasites show an abnormal morphology *in vitro*

For detailed information about the morphology of the mutant parasites, live imaging experiments were performed, where *Pb*Sar1(H74L) parasites were compared to *Pb*Sar1 parasites. The general appearance of the parasites was regarded as well as the shape of nuclei and cytoplasm (see Fig. 4.3.1).

At 24 hpi, both *Pb*Sar1 and *Pb*Sar1(H74L) parasites showed GFP evenly distributed throughout the cytoplasm and a single, often multi-lobed nucleus. At later liver stages, however, eye-catching differences were seen. *Pb*Sar1 parasites showed vast numbers of nuclei, implying many rounds of nuclear division, whereas the number of nuclei in *Pb*Sar1(H74L) parasites was drastically reduced. This abnormal development was demonstrated additionally by a larger size of these nuclei. As the secretory pathway in *Pb*Sar1(H74L) parasites is disturbed, the reason for an insufficient nuclear division could be in a lack of nutrients, particularly missing nuclear acids.

Differences were also present in the appearance of the cytoplasm. *Pb*Sar1 parasites reach the phase of plasma membrane invagination at 54 hpi, resulting in areas lack-

ing GFP staining due to the subdivision of the cytoplasm. Instead, *Pb*Sar1(H74L) parasites frequently showed vacuole-like structures, where the cytoplasmic GFP staining is absent. This phenomenon was rare in the control and if present was less distinct, with small numbers of control parasites showing very few, very small vacuoles. The origin of these vacuoles remains unexplained, it could be lipid droplets, autophagosomes or any kind of endosomes. However, it is a sign of parasites that will ultimately die rather than maturing to form merozoites.

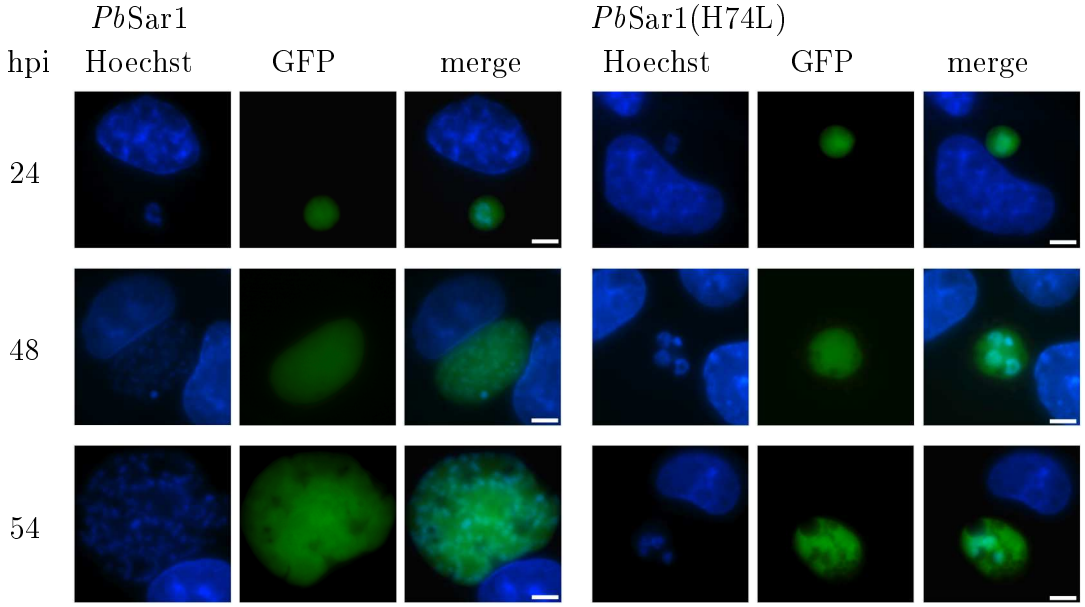

Figure 4.3.1: Live imaging at 24, 48 and 54 hpi

Both host cell and parasite nuclei are stained with Hoechst (blue): parasite cytoplasm is filled with GFP (green). At 24 hpi, no difference in the morphology is observed. At 48 hpi, *Pb*Sar1 parasites show a much larger size than at 24 hpi, with a vast number of nuclei and solid GFP staining. *Pb*Sar1(H74L) parasites, in contrast, are less increased in size and show very few but larger nuclei. At 54 hpi, differences in the size of the parasites and number of nuclei are conspicuous. *Pb*Sar1(H74L) parasites present many vacuole-like structures inside the cytoplasm that lack GFP-staining. Scale bar: 10 µm

Regarding this abnormal GFP distribution in the cytoplasm of *Pb*Sar1(H74L) parasites, further experiments were performed to quantify this phenomenon. Cells infected with *Pb*Sar1 and *Pb*Sar1(H74L) parasites were fixed at 24, 48 and 54 hpi, stained with anti-GFP antibodies and analysed for this phenomenon. Again,

many vacuole-like structures with no GFP staining were present in *Pb*Sar1(H74L) parasites at later time points. The GFP distribution in the cytoplasm of these parasites was reminiscent of Swiss cheese. To quantify this phenomenon, the number of parasites with such an abnormal distribution in the cytoplasm was counted (see Fig. 4.3.2). More than twice as many *Pb*Sar1(H74L) parasites showed vacuole-like structures, increasing in later timepoints.

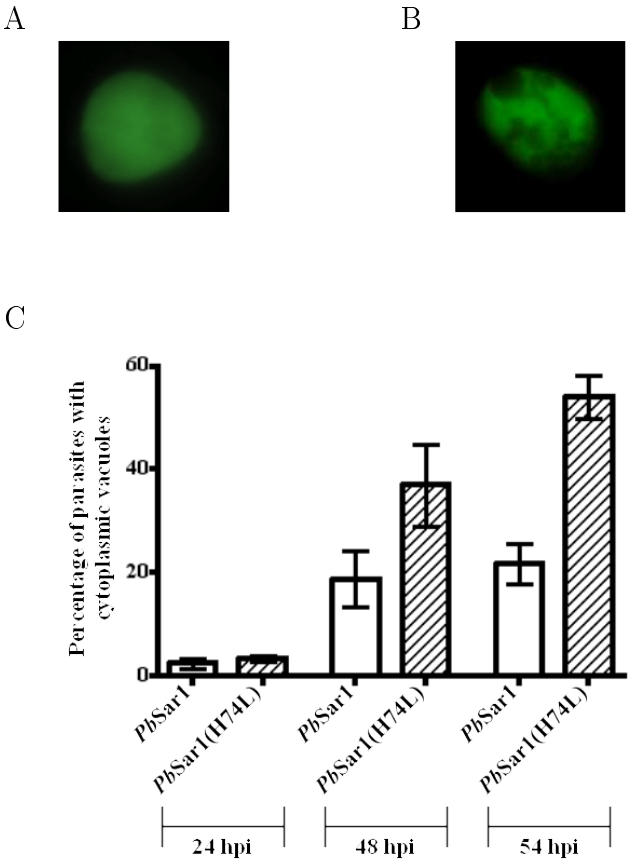

Figure 4.3.2: Quantification of cytoplasmic vacuole-like structures.
Cells were fixed and stained with anti-GFP antibodies (green). Parasites with and without vacuole-like structures were counted. A: Parasite with normal GFP staining. B: Parasite with vacuole-like structures inside the cytoplasm, from which GFP is missing. C: Percentage of parasites with vacuoles inside the cytoplasm at different time points. *Pb*Sar1(H74L) parasites much more frequently show an abnormal GFP distribution in the cytoplasm, as seen by the presence of vacuoles.

4.4 Sar1 GTPase(H74L)-expressing parasites show abnormal Exp1 trafficking to the PVM

As the mutation H74L of the Sar1 GTPase interferes with the protein transport of the secretory pathway, further experiments were performed to localise proteins normally transported to the PVM, presumbly via the secretory pathway. Exp1 is a transmembrane protein expressed during liver stage development and normally transported to the PVM of the parasite (Sanchez et al., 1994). To examine defects in protein transport, cells were fixed at 48 and 54 hpi and Exp1 was localised with an anti-Exp1 antibody. Knowing the promoter lisp2 starts its activity at approximately 24 hpi, studies were concentrated on later liver stages. Staining with anti-Exp1 antibodies in immunofluorescence assays normally labels the PVM of *Plasmodium* parasites during the liver stage and is typically seen as a complete ring surrounding the parasite.

To identify infected cells easily, additional staining with anti-GFP antibodies and DAPI was performed (see Fig. 4.4.1). The vast majority of parasites expressing the wildtype form of Sar1 GTPase, *Pb*Sar1, showed normal Exp1 staining, whereas *Pb*Sar1(H74L) parasites were often only partly surrounded by an Exp1 ring or Exp1 was absent outside of the parasite This phenomenon can be interpreted as an indication that there is a defect in the protein transport apparatus of the parasite.

Occasionally, in the later stage at 54 hpi, GFP was seen not only restricted to the cytoplasm of the parasite but also within the host cell's cytoplasm. Interestingly, this phenomenon was only found very occasionally in *Pb*Sar1, in contrast to *Pb*Sar1(H74L) parasites where it occurred frequently.

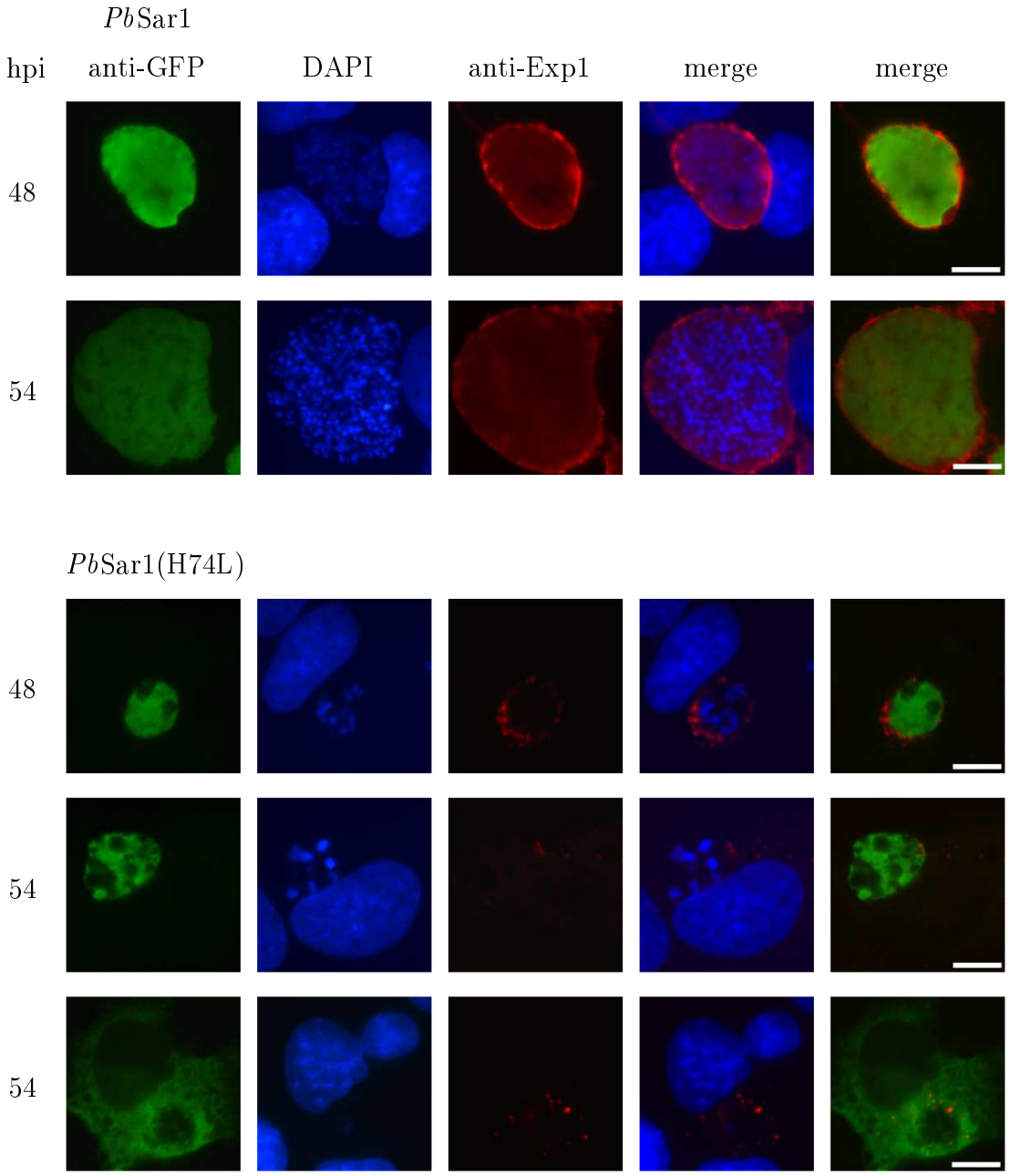

Figure 4.4.1: Immunofluorescence assays at 48 and 54 hpi, staining with anti-GFP antibodies (green), DAPI (blue) and anti-Exp1 antibodies (red).
At both timepoints, *Pb*Sar1 parasites show a normal size, a normal GFP distribution in the cytoplasm and large numbers of nuclei. Exp1 localises to the PVM, which surrounds the parasite completely. *Pb*Sar1(H74L) parasites, in contrast, show a smaller size, an abnormal GFP distribution with vacuoles and fewer nuclei. Only a partial PVM, or a complete PVM only partially labeled with Exp1 is seen at 48 hpi, whereas at 54 hpi, Exp1 staining is completely absent. Lower row: GFP is not restricted to the parasite but has leaked through the PVM and fills the host cell's cytoplasm. Scale bar: 10 µm.

To quantify both, the different Exp1 pattern and parasites with a GFP-filled host cell, parasites were counted at 48 and 54 hpi and grouped into several categories (see Fig. 4.4.2): a complete surrounding of the parasite by Exp1 staining, only partly surrounding, no Exp1 staining or only a few dots and a GFP-filled host cell, where Exp1 staining is always absent.

At both timepoints, most of the *Pb*Sar1 parasites showed a normal Exp1 staining, whereas in *Pb*Sar1(H74L) parasites, a normal staining was very rare. At 48 hpi, the vast majority of PbSar1(H74L) parasites showed an abnormal or no Exp1 staining and at 54 hpi, the number of *Pb*Sar1(H74L) parasites with no Exp1 staining increased. Additionally the numbers of parasites with a GFP leakage into the host cell rose.

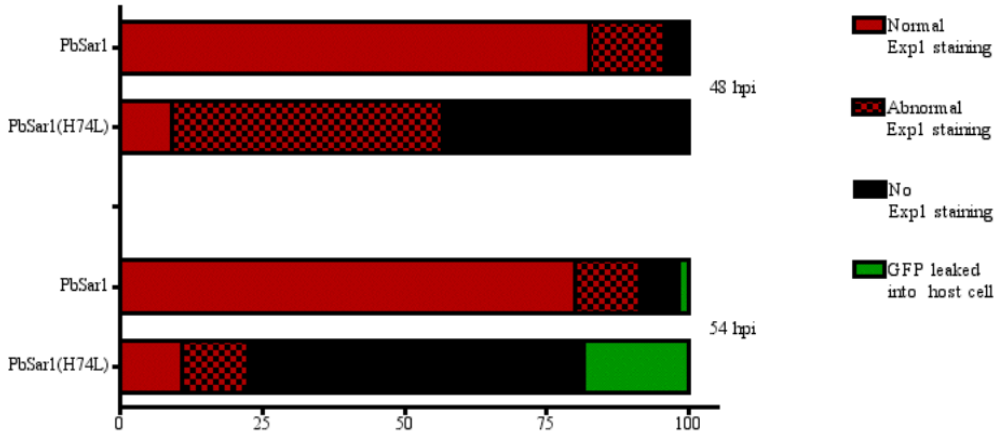

Figure 4.4.2: Quantification of Exp1 staining

For quantification, IFAs with anti-Exp1 antibodies of *Pb*Sar1 and *Pb*Sar1(H74L) parasites at 48 and 54 hpi were grouped: Red: PVM is stained completely with anti-Exp1. At both timepoints, more than 80% of *Pb*Sar1 parasites show a normal Exp1 staining; in *Pb*Sar1(H74L) parasites less than 10%. Red and black checked pattern: incomplete ring of Exp1 staining surrounds the parasite. At 48 hpi, 49% of the *Pb*Sar1(H74L) parasites show an abnormal Exp1 staining compared to 18% of the *Pb*Sar1 parasites. Black: No Exp1 staining or only a few dots appear at the PVM. At both timepoints, a large percentage of *Pb*Sar1(H74L) parasites do not show Exp1 staining. Green: GFP is not restricted to the parasite but inside the host cell's cytoplasm. This phenomenon is only seen at the later time point and is very rare in *Pb*Sar1 parasites. In contrast, almost 20 % of *Pb*Sar1(H74L) show GFP leakage into the host cell.

5 Discussion

The intracellular development of *Plasmodium* parasites during liver stage is a challenge as hepatocytes have the capacity to fight against pathogens. They are able to kill parasites directly (Yano and Kurata, 2011). If this fails, hepatocytes have the ability to induce apoptosis to eliminate the parasite from the organism. In addition, the immune system can induce a programmed cell death. Despite this, during evolution *Plasmodium* parasites have developed the capacity to survive and develop smoothly, assuming a well established secretory system for interaction with the host cell. This study analyses the role of protein transport for the parasite as well as the reaction of the host cell, when the parasite is hindered in suppressing the normal defence mechanisms of the hepatocyte.

The liver stage-specific promoter lisp2 allows the controlled expression and downstream analysis of defined genes exclusively during the liver stage (Helm et al., 2010). Recently, De Niz *et al.* could confirm the liver stage-specificity also *in vivo*. This promoter starts its activity during the liver stage at 24 hpi, with a maximum activity at 54 hpi; it is not active in blood or mosquito stage (DeNiz et al., 2015). The lisp2 promoter region normally regulates the transcription of the corresponding protein LISP2, a protein localising to the PVM as well as to the cytosol and nucleus of the host cell and thought to play a key role in interacting with the hepatocyte (Orito et al., 2013).

As Sar1 is involved in the initial steps of secretory transport and as there is already

evidence for serious effects when a dominant negative mutant of Sar1 GTPase is expressed in yeast and plants (Saito et al., 1998; Takeuchi et al., 2000), this was chosen as a protein of interest. This study demonstrates the importance of the secretory pathway for the liver stage development of *Plasmodium* parasites.

In other systems, mutation of a conserved histidine residue at approximately position 74 into a leucine leads to an insensitivity of Sar1 to its GAP and consequently to a fixed GTP-bound state (Saito et al., 1998). The increasing activity of the promoter should lead to an accumulation of mutated Sar1, which would compete with the endogenous protein, rendering most or all Sar1 GTPase molecules inactive. This should lead to an inhibition of protein transport from the ER to the Golgi (Takeuchi et al., 2000). Consequently, secretion of proteins for interaction with and manipulation of the host cell would be restricted. With this lack of secretory pathway in later stages of development, when the promoter activity is stronger, parasites were expected to be blocked in their development. The strong effects on the phenotype imply a major role for *Plasmodium* liver stage development.

Phenotype of Sar1 GTPAse(H74)-expressing paraites

The assessment of parasite developmental success was on the basis of size and morphology such as GFP localisation and nuclear development plus changes in the PVM. As the promoter is not active before 24 hpi (Helm et al., 2010), normally developed parasites at least until this timepoint were expected. As predicted, there was no significant difference between the size of mutated and wildtype parasites at the early time point of 24 hpi. This was true for both the size and the morphology: Parasites showed an even cytosolic GFP staining and one single nucleus. In contrast, in the later liver stages, parasites overexpressing the mutant form of Sar1 GTPase, *Pb*Sar1(H74L), showed a drastically reduced size and an abnormal morphology. In addition, the smaller parasites showed abnormal looking nuclei:

they did not divide as often, instead showing a bigger size. This phenomenon was seen several times by Stanway *et al.*, when parasites were disturbed in their development by drug treatment or genetic manipulation (data not published). This can be assumed as a general sign of lack of parasite fitness. Here, the reason for lack of fitness could be the lack of nutrients such as nucleotides and membrane components for the nucleus, potentially due to a lack of uptake transporters delivered to the plasma membrane or PVM.

Exp1 is a membrane protein, a glutathione S-transferase, located in the PVM (Lisewski et al., 2014). Parasites stained with anti-Exp1 antiserum show a bright ring surrounding the parasite completely. Parasites overexpressing the wildtype form of Sar1 GTPase, *Pb*Sar1, mostly presented this normal pattern of staining within the PVM. *Pb*Sar1(H74L) parasites, in contrast, mostly showed little or no Exp1-staining within the PVM. A decrease of Exp1 presented in the PVM is not surprising as the protein transport is hindered. The correctly produced Exp1 is not carried to the Golgi because of the lack of the COPII vesicle function. Consequently, transport to the PVM fails. However, in cases where little Exp1 was present surrounding the parasite, there rarely seemed to be an increase in the levels of Exp1 within the parasite cytoplasm. One would assume that the production of proteins in the ER is not compromised. Is there a reduced transcription and translation of Exp1? This could be due to a general inhibition of parasite development and a compromise in parasite fitness after about 24 hpi, causing only little Exp1 to be produced. It could also be that the antibodies do not bind because of the missing modification process inside the Golgi network. This could be verified by further studies with an additional insertion of an mCherry-tagged Exp1 in *Pb*Sar1(H74L) parasites. With this, the intracellular trafficking of Exp1 could be followed by live imaging.

Why does *Pb*Sar1(H74L) parasites form so few detached cells?

Normally, at 72 hpi, detached cells and merosomes have already been formed (Sturm et al., 2006). In contrast to *Pb*Sar1 and *Pb*GFP parasites, which showed a normal conversion rate (about 18 % and 24 %), the conversion rate of *Pb*Sar1(H74L) parasites was less than 1 %. The detachement of the host cells is a result of a parasite-dependent host cell death (Heussler et al., 2010): The host cell membrane stays intact but rounds up and loses its fixation to the plates surface *in vitro*. The trigger for parasite-dependent host cell death and therefore detachment of the cells is the rupture of the PVM (Heussler et al., 2010). Until now, the molecular process of the PVM breakdown is not understood completely. A protein known to be involved in the breakdown of the PVM is LISP1 (Ishino et al., 2009), a membrane protein thought to be transported together with the protein LISP2 to the PVM via secretory pathway (Orito et al., 2013). The trigger of it still remains unexplored. However, one could postulate that the complete assembly of merozoites is one condition.

Besides the successful amplification and segregation of the nucleus, apicoplast and mitochondrion, all secretory organelles have to be formed *de novo* (Kats et al., 2008). Proteins destined for the rhoptries make their way via the secretory pathway (Kats et al., 2006). Considering the disturbance of this pathway in *Pb*Sar1(H74L) parasites, not only is nutrition likely lacking but secretory organelles can presumably not be formed (Jaikaria et al., 1993; Kats et al., 2008). Without these essential organelles, the formation of mature merozoites would be inhibited. In addition, the protein LISP1, known to be involved in PVM disruption, is assumed to not be transported to the PVM. It can be postulated that the reduced conversion rate is caused by several disabilities: the incomplete assembly of merosomes on one side and the insufficient enrichment of LISP1 at the PVM on the other side. Further studies with anti-LISP1 antibodies are necessary to prove this thesis.

Can host cell death not be prevented anymore?

Almost 20 % of the *Pb*Sar1(H74L) parasites showed GFP-staining also inside the host cell at 54 hpi, compared to less than 1 % in the *Pb*Sar1 parasites. As this phenomenon is consistent with an incomplete surrounding of the parasite by an Exp1-stained PVM, it can be assumed that these parasites have problems to maintain an intact PVM and plasma membrane, resulting in a GFP leakage and dissemination in the host cell cytoplasm.

Normally, parasites avoid apoptosis of their host cell by secretion of apoptosis-inhibiting factors (van de Sand et al., 2005). Deficiencies in secretion would clearly lead to an insufficient repression of host cell apoptosis. Do *Pb*Sar1(H74L) parasites lose their ability to prevent host cell apoptosis? Interestingly, no host cell DNA fragmentation or cell shrinkage, normal signs of apoptosis, were seen. In contrast, host cells containing GFP inside the cytoplasm maintained a seemingly normal morphology, remaining connected to neighbouring cells and to the cell culture dish. It is possible that the avoiding of apoptosis is already induced during the invasion process and early liver stage and therefore a later handicap in protein secretion has no effect on this process.

If apoptosis is still prevented, what else happens in a host cell containing a compromised parasite? Occasionally, HepG2 cells were seen that did not contain an obvious parasite, but that showed very pale GFP staining (data not shown). It can be assumed, these are older stages of the above described process: The parasite's membrane and PVM have broken down, GFP has leaked through the host cell's cytoplasm and the parasite has been cleared out, so that it was no longer visible. It seems, that the host cell is able to eliminate the parasite without itself being destroyed.

Is there parasite autophagy going on?

Another unexpected phenomenon during the later liver stages was the presence of vacuole-like structures inside the parasite's cytoplasm, that lacked GFP-staining. Although this was observed occasionally in *Pb*Sar1 parasites, too, it was much more distinct in *Pb*Sar1(H74L) parasites. So far, it can only speculated about the nature of these vacuoles:

One idea is the development of autophagosomes. Autophagy is a cellular process that occurs in eukaryotic cells to support survival in phases of starvation or to remove redundant cellular materials. Mutated parasites reach a phase of starvation due to a block in nutrient uptake, leading to the formation of autophagosomes. If *Plasmodium* parasites indeed use this strategy and if the parasites die an autophagic cell death, still needs to be elucidated. In the recent published work of Eickel *et al.*, coding sequences for homologues of typical marker proteins of autophagy were found in the parasite's genome and autophagosome-like vesicles with multiple membranes were seen in electron microscopy analysis in dying parasites. Similar to this study here, the vesicles shown by Eickel *et al.* were also localised near the parasite membrane. However, marker proteins of autophagy did not colocalise with the autophagosome-like vesicles (Eickel et al., 2013).

Additionally, it has been shown that both GTPases Rab1b and Sar1 are necessary for autophagosome formation: In experiments from Zoppino *et al.* with Chinese hamster ovary cells expressing a mutated Sar1 GTPase, autophagy was induced by incubation in starvation medium but autophagosomes could not be visualised (Zoppino et al., 2010). In their study, the performed mutation of Sar1 resulted in the GTP-bound form, the same state used in this study. As such a mutation of Sar1 GTPase hinders the formation of autophagosomes, it is not very likely that the vacuoles seen in this study are autophagosomes. It remains possible, however, that the molecular basis of autophagy in *Plasmodium* parasites differs

from other euakryotes. It is imaginable, that Sar1 GTPase does not play a role in autophagosome formation in *Plasmodium* parasites.

What else could induce the formation of these vacuoles? Interestingly, the vacuole-like structures do not contain GFP. It could be that GFP has already been degenerated and has lost its fluorescence, as would occur inside autophagosomes. The vacuoles could also potentially originate from the host cell cytoplasm, where no GFP is present. Actually, many of these vacuoles are found near the plasma membrane. Is it possible that these structures are kind of endosomes? The process of how larger molecules are transported across the two membranes is not yet elucidated completely. However, Orito *et al.* found LISP2-filled vesicles at the PVM, suggesting these as the last step of the secretory pathway (Orito et al., 2013). If cargo is transported through both membranes within vesicles outside the parasite, the opposite way is also imaginable. Another explanation is the leakage of the PVM, resulting in influx of host cell material in the intermembrane area, afterwards pushed into the parasite, enclosed by the PM.

Why do *Pb*Sar1(H74L) expressing parasites survive anyway?

Parasites with a disturbed secretory system are hindered in a proper manipulation of their host cell, which can lead to several problems: Firstly, parasites are restricted in their ability to achieve sufficient nutrient uptake. Secondly, the prevention of host cell death could fail. Thirdly, the prevention of the activation of the cytosolic immune reaction and host immune system *in vivo* can be disturbed and rather lead to an elimination of the parasite from the host cell. Considering these facts, it is remarkable, that some Sar1(H74L) parasites survive.

One reason is the delayed start of the promoter's activity at 24 hpi (Helm et al., 2010). Until then, the parasite has enough time to produce sufficient wildtype Sar1 GTPase for some time. When the promoter starts to be active and mutated

Sar1 GTPase is transcribed, competition with wildtype Sar1 begins and increases. Even if huge amounts of mutated Sar1 GTPase is produced, few amounts of wildtype Sar1 GTPase will still be there and can achieve at least part of the protein transport. Particularly, only small amounts of Sar1 GTPase are necessary for the fission of the coated vesicles, the process blocked with the H74L mutation.

A second reason is the preparation of the parasite for the liver stage already during sporozoite stage: During the process of invasion of *Apicomplexan* parasites into their host cell, it is likely that many proteins get secreted from secretory organelles (Mota and Rodriguez, 2002). Although in *Plasmodium* parasites most research of the molecular invasion process occurs during the blood stage, it can be assumed that the process of invasion of sporozoites is at least similar to the process of invasion of merozoites. Proteins stored in rhoptries are secreted in a special order (Kats et al., 2008): The first portion is essential for the invasion itself (for example formation of tight junctions), the second portion is used for modifications of the host cell and the last portion is needed for upkeeping the PV during intracellular stage. Hence, many processes like prevention of host cell's death or hiding from the immune system behind a host cell derived PVM are already induced during the invasion process or even before: This is true for UIS4, a protein already translated and transported during the sporozoite stage but designated for secretion in liver stage (Aly et al., 2008; Mueller et al., 2005). It is a transmembrane protein which localises to the PVM (Aly et al., 2008). Its function is speculated to play a role in absorbing nutrients. Proteins like this can be one reason that *Pb*Sar1(H74L) expressing parasites survive anyway.

Prado *et al.* showed that hepatocytes attack the invading parasite by marking it with LC3, an autophagy marker protein. Although this can be understood as a defence strategy, which indeed eliminates some parasites, surviving parasites seem to benefit from this initial attack and use it as an additional source of amino acids

(Prado et al., 2015). Parasites with a disabled secretory system may be destroyed more often by autophagy of the host cell. On the other hand, parasites still able to survive this attack could hereby compensate their lack.

Are *Pb*Sar1(H74L) parasites able to infect red blood cells *in vivo*?

Even if a few detached cells were seen in *Pb*Sar1(H74L) parasites, it is possible that the merozoites inside are not infective. As described above, secretory organelles have to be formed *de novo* and filled with proteins via the secretory pathway. These organelles could be empty or filled insufficiently, leading to merozoites not able to invade a red blood cell.

One of the problems of interpreting the results is the fact that the transfected parasites are not clonal. It is possible that different parasites may have different numbers of gene copies due to the presence of one or more episomal plasmid copies and therefore the appearance of the phenotypes may vary. To verify if the expression of the H74L mutant of Sar1 GTPase can lead to a phenotype strong enough that merozoite formation and thus clinical malaria is avoided completely, can only be established by having a clonal population of parasites and subsequently using sporozoites from these parasites to infect mice. For this, it would be necessary to introduce the Sar1 GTPase expression cassettes into the genome by double homologous recombination to prevent the possibility of reversion to wild type, which can remain an issue despite cloning of parasites if integration only occurs by single homologous recombination, as is the case for the pL0017.1.7 plasmid.

To proof, whether *Pb*Sar1(H74L) parasites are able to form infective merozoites *in vivo*, further studies with clonal parasites would be necessary.

6 Conclusion

A promising tool for shedding light on molecular events during the *Plasmodium* liver stage is the recently described liver stage-specific promoter lisp2 (formally called LSA4) (Helm et al., 2010; DeNiz et al., 2015). The liver stage-specific promotor lisp2 can be used to successfully drive the expression of a dominant-negative mutant protein at a strength that leads to abnormal parasite development. Overexpression of the mutant Sar1(H74L) hinders parasite maturation, leads to morphological abnormalities and appears to prevent secretion of proteins. It has been shown that the lisp2 promoter can be used for investigate proteins and that the principle of dominant negative mutant protein expression works successfully in *Plasmodium*. A similar approach could therefore be used in the future to investigate protein function during liver stage development or even to generate parasites that fully arrest during the liver stage, offering sterile protection against subsequent infection with viable parasites. It has been shown that the viability of *Plasmodium* parasites can be disturbed exclusively in the liver stage with the tool of a dominant negative mutant protein which is expressed under the control of the strong liver stage-specific promoter lisp2. *P. berghei* parasites expressing the mutant form of the Sar1 GTPase are restricted in their development, thus drastically reduced in their capability to form merosomes. Parasites overexpressing this mutant protein show a reduced growth rate, an abnormal morphology and have a greatly reduced conversion rate from mid to mature liver stages.

7 Zusammenfassung

Der Leberphasen-spezifische Promotor lisp2 (zuvor LSA4 genannt) kann für die Erforschung der molekularen Abläufe der Leberphase von *Plasmodien* genutzt werden (Helm et al., 2010; DeNiz et al., 2015). Ein dominant-negativ mutiertes Protein, welches unter diesem Promotor stark exprimiert wird, kann zu fehlentwickelten Parasiten führen. Die in dieser Arbeit gewählte Überexpression der Mutante Sar1(H74L) führt zu Reifungsstörungen der Parasiten und diversen morphologischen Veränderungen. Die Proteinsekretion scheint hierbei unterdrückt. Parasiten der Spezies *P. berghei*, welche die mutierte Form der Sar1 GTPase exprimieren, sind in ihrer Entwicklung eingeschränkt und daher auch in ihrer Fähigkeit gehemmt, Merosomen zu bilden. Sie zeigen ein gehemmtes Wachstum, eine abweichende Morphologie sowie eine deutlich geminderte Konversionsrate von der mittleren zur späten Leberphase. Es konnte gezeigt werden, dass das Prinzip der Expression einer dominant negativen Mutante bei *Plasmodien* erfolgreich funktioniert. Darüber hinaus konnte gezeigt werden, dass mit dieser Methode die Lebensfähigkeit der *Plasmodien* ausschließlich während der Leberphase gestört werden kann, sofern dies unter der Kontrolle des Leberphasen-spezifischen Promotors lisp2 geschieht. In Zukunft könnte ein ähnlicher Ansatz genutzt werden, um die Funktion von Proteinen während der Leberphase zu untersuchen. Möglicherweise könnten Parasiten erzeugt werden, deren Entwicklung während der Leberphase komplett gestoppt wird, was einen nachfolgenden Infektionsschutz bieten könnte.

8 Abbreviations

%	percentage
°C	degrees Celsius
™	trade mark
®	registered
©	copyright
A	Austria; adenine
Amp	Ampicillin
ATP	adenosine triphosphate
B	Belgium
BNI	Bernhard-Nocht-Institute
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CH	Switzerland
cm	centimetre
CO_2	carbon dioxide

COPII	Coat protein complex II
CSP	circumsporozoite protein
CV	coated vesicle
D	Germany
Da	Dalton
DAPI	4'6-diamidino-2-phenylindol
dATP	deoxyadenosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
ECCC	European Collection of Cell Cultures
EDTA	ethylene diamine tetraacetic acid
ef1α	eukaryotic elongation factor 1 alpha
ER	endoplasmic reticulum
et al.	*et alii*
Exp1	exported protein 1
FBS	foetal bovine serum
FCS	foetal calf serum
g	gram; gravitational force
G	guanine
GAP	GTPase activating protein
gDNA	genomic DNA

GDP	guanosine diphosphate
GEF	GTP exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
H	histidine
HBsAg	Hepatitis B surface antigen
HepG2	hepatocellular carcinoma line G2
HF	High Fidelity
hpi	hours post infection
I	Italy
IFA	immunofluorescence assay
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobases
l	litre
L	leucine
LB	lysogeny broth
LISP1	liver stage-specific protein 1
lisp2	liver stage-specific promoter 2
LISP2	liver stage-specific protein 2
LSA4	liver stage-specific antigen 4
M	molar
MEM	minimum essential medium

$MgCl_2$	magnesium chloride
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
mol	mole
MSP1	major surface protein 1
Na_2HPO_4	disodium phosphate (sodium hydrogen phosphate)
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
ng	nanogram
NL	Netherlands
NMRI	Naval Medical Research Institute
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
*Pb*ICB	*P. berghei* inhibitor of cysteine proteases
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	potential of hydrogen
PM	parasite membrane
pmol	picomole
PV	parasitophorous vacuole

PVM	parasitophorous vacuole membrane
s	second(s)
SeqLab	Sequence Laboratories Göttingen GmbH
T	thymine
TAE	Tris base, acetic acid, EDTA
TNF-α	tumor necrosis factor alpha
U	units
UIS 3/4	upregulated in infectious sporozoites 3/4
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volt
WHO	World Health Organisation
x	times
µg	microgram
µl	microliter

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10 Curriculum vitae

Personal data

- Name (first, middle, second): Anke Waltraud Ute Schlüter
- Date and place of birth: 02 September 1982 in Backnang (Germany)

Educational career

- 1989 - 2002: Primary and Grammar School (Winnenden)
- 2003 - 2005: University of Hamburg: Physics (Intermediate Diploma)
- 2005 - 2006: University of Göttingen: Dentistry
- 2006 - 2007: University of Magdeburg: Medicine (preclinical part)
- 2007 - 2012: University of Hamburg: Medicine (clinical part)

Official qualifications

- Abitur: Grade 1.9 (June 2002)
- Physics: Intermediate Diploma: Grade 1 (March 2005)
- Medicine: state examination: Grade 1 (September 2007)

Professional career

- 07/2012 - 07/2016: medical assistant in Albertinen Krankenhaus Hamburg.
- since 08/2016: medical assistant in the medical practice of Horst Schlüter (general practitioner) in Winnenden.

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