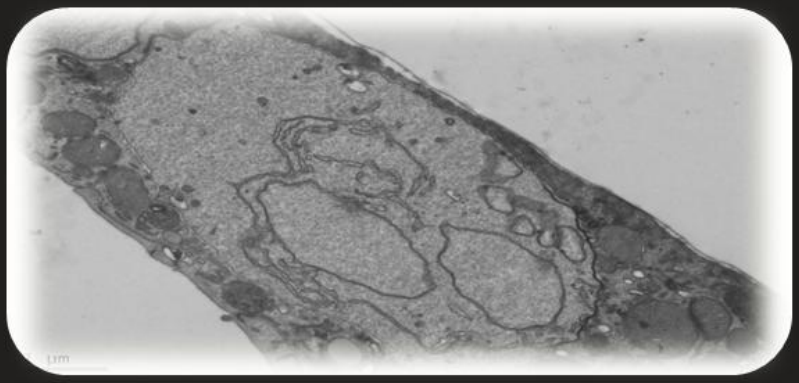
Dissecting the molecular interaction between hepatocytes and *Plasmodium* liver parasites

Mafalda Lopes da Silva



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

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Research work coordinated by:

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Preface

The work presented in this thesis is the result of the work developed during my Ph.D. research project.

The experimental work was developed mainly at Instituto Gulbenkian de Ciência, in Oeiras, while the siRNA screen, described in Chapter 3 was performed at Imperial College London. All work was supervised by Professor Miguel C. Seabra.

Financial support was provided by Fundação para a Ciência e Tecnologia. Portugal, through the Ph.D. fellowship grant SFRH/BD/27705/2006.

This thesis is structured in 5 Chapters.

Chapter 1 compromises a general introduction to malaria, with a focus on the liver stage of the *Plasmodium* life cycle. A general review of well-known examples of other intracellular host-pathogen interactions is described, as well as the objectives of this thesis.

Chapter 2 characterizes the possible host-*Plasmodium* interactions during the liver stage of infection.

Chapter 3 shows the results obtained during the siRNA screen of host trafficking proteins and their effect on *Plasmodium* infection.

Chapter 4 further characterizes the role of the host-*Plasmodium* interactions found in Chapter 2.

Each of these results chapters includes a Summary, the relevant Materials & Methods used and a Discussion.

Chapter 5 includes a general discussion of the results obtained, their relevance, and future perspectives.

Acknowledgements

First of all, I would like to thank Miguel, my supervisor, first, for believing from the very beginning that I was able to complete a PhD. Secondly, for being the eternal optimist and motivator while understanding that, at times, being at the "bench" isn´t easy. And at times, it wasn´t! And for being present, even when he physically wasn't.

Secondly, I would like to thank Carolina, for her positive attitude, even on my worst days, and for caring, even if it's not her experiment. I definitely would have not done half of what I did without you!

I also want to thank Elsa and Laura. Elsa for all the support during the first years, when it was just the two of us in the group, and Laura, more recently, for sharing her expertise of the Malaria world.

And to the rest of the group at the IGC and FCM, with a special thank you to Zé for all the "help" with constructs, and to everyone else, for their productive comments and suggestions during lab meetings.

I also want to thank Ligia, for sharing her know-how in malaria but also for taking time to just talk about anything, especially when I needed it the most. And for the patience of teaching me how to isolate primary mouse hepatocytes.

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A special thanks to Dr. Sinden and Rebecca, at Imperial College, for their excellent technical help and availability to hear my story and give productive suggestions.

A big thank you to the entire Malaria group at IMM, special Maria and Cristina. Maria, for all the technical help, mosquitos and more importantly, her availability to contribute to my work. Cristina, for teaching me in the beginning, all I knew about malaria, when I knew nothing at all.

A very special thank you to all the people (past and present) in the IGC imaging facility; Nuno Moreno, Ricardo Henriques and Franscisco. All that I know about microscopes, is their fault! And for having the patience to fix problems when they arose, even if during “inappropriate” hours. Thank you!

I would also like to thank Professor Coutinho, for being my adoptive supervisor in the beginning, and allowing me to attend his labmeetings during my first months at the IGC. And to all the people (past and present) in the wider IGC community. For always being available to share their knowledge (and reagents!) and often taking time to teach me new things, when they didn´t have too.

And finally, I would like to thank all my friends and family, especially my mother, father and grandmother. For listening to me after all the bad days in the lab, and motivating me to go back the next day. And for also supporting me through the good times, and being happy when experiments worked, even when they didn´t understand what I was doing! I would have finished this PhD without you!

Acronyms and Abbreviations

AMA1 - apical membrane antigen 1
ATG - autophagy related genes
cAMP - cyclic adenosyl monophosphate
CBD - cholesterol binding domain
CelTOS - cell transversal protein for ookinetes and sporozoites
COPI – coat protein complex
CSP - circumsporozoite protein
EEA1 - early endosome antigen 1
EEF - exo-erythrocytic form
ER - endoplasmic reticulum
FASII - type II fatty acid synthesis
GAP - GTPase activating protein
GAS - genetically attenuated sporozoites
GDF - GDI-displacement factor
GEF - Guanine nucleotide exchange factor
GTPase- guanosine triphosphates
HGF - hepatocyte growth factor
HSPG - heparin sulphate proteoglycans
LAMP1 - lysosome associated membrane protein
LC3 - microtubules associated protein 1 light chain 3
L-FABP - liver fatty acid synthesis
LISP1 - liver specific protein 1
LPG - lipophososphoglycan
MVB – multi vesicular bodies
PI(4)P - phosphatidylinositol 4-phosphate
PL - phosoholipase
PVM - parasitophorous vacuole membrane
RAS - radiation attenuated sporozoites
RBC - red blood cell
RILP - Rab interacting lysosomal protein

ROP2 - rhoptry protein 2

SAP1 - sporozoite asparagines rich protein 1

SERA - serine repeat antigens

SNARE - Soluble N-ethylmaleimide-sensitive factor activating protein receptor

SR-BI - scavenger receptor type B I

SPECT - sprozoite microneme protein essencial for cell transversal

TRAP - thrombospin related anonymous protein

UIS - upregulated in infectious sporozoites

vATPase – vacuolar proton pump ATPase

VPS34 - vacuolar sorting protein 34

List of Figures and Tables

Summary

Malaria is one of the world´s leading causes of death, responsible for over 700,000 deaths per year, the majority of which are African children under 5 years of age. Malaria disease is caused by the transmission of an Apicomplexa parasite, *Plasmodium*, through the bit of a female *Anopheles* mosquito, and transmitted parasites quickly reach the mammalian host liver, where the first round of replication begins.

Plasmodium sporozoites, once inside the liver, must invade and survive within hepatocytes until the first replicative stage within the mammalian host is accomplished. Upon migration through various cells, sporozoites are able to actively enter hepatocytes, forming a Parasitophorous Vacuole Membrane (PVM) around itself. Once this intracellular niche is established, parasite replication and growth is initiated. Dramatic morphological as well as gene expression modifications occur at this stage, and the parasite achieves one of the highest replication rates known within eukaryotic species (Sinnis and Sim, 1997).

Although the *Plasmodium* life-cycle has been extensively characterized, relatively little is known about sporozoite interaction with host organelles, vesicles and proteins. To address this issue, *Plasmodium* interactions with the host cell endomembranes was analyzed at various stages of liver infection using indirect immunofluorescence. *Plasmodium* parasites were seen closely associated with host endoplasmic reticulum (ER) and the Golgi apparatus. Surprisingly, late endosomes/lysosomes, observed with the membrane markers Rab7a, CD63 and LAMP1, aggregated around the parasite. No interaction with host peroxisomes, early and recycling endosomes was observed.

To complement and extend the localization studies, the effect of depleting Rab GTPases, regulators of organelle identity, on parasite liver infection rate was investigated using a siRNA library. Only depletion of Rab1a

significantly affected liver infection rates showing a marked increase in infection. Rab1a has been implicated in ER-to-Golgi trafficking and more recently, in initial autophagosome formation in autophagy, a process by which host cytoplasmic material is degraded and recycled in response to stress. Autophagy also plays a role in antibacterial destruction and elimination, and thus, various pathogens have evolved mechanisms to avoid autophagic elimination.

Further characterization of the role of Rab1a revealed its function in autophagy, rather than ER-Golgi trafficking, affected *Plasmodium* liver infection. More importantly, Rab1a seems to be important during the initial stages of development by enhancing host parasite elimination, since its depletion leads to an increase in parasite infection rate. During the later stages of infection, Rab1a may be required for parasite replication, since exo-erythrocytic forms (EEFs) are smaller in Rab1a depleted cells. Colocalization with the autophagic marker LC3 revealed that LC3 vesicles aggregate very strongly around the parasite and seems to be upregulated in infected cells suggesting a novel and important role for autophagy during *Plasmodium* liver infection.

Further characterization of the identity of vesicles that aggregate around *Plasmodium* parasites revealed that they are negative for Cathepsin D, a hydrolytic enzyme present on degradative lysosomes and acidic as determined by utilizing pH sensitive dyes. To study the role of these acidic vesicles, acidification of vesicles was disrupted using Concanamycin A which inhibits vATPase and ammonium chloride which dissipates the pH gradient within cells. Both treatments inhibit the trafficking and maturation of late endosomes, MVBs, amphisomes and lysosomes and had no effect on parasite infection rate but severely impaired parasite growth, especially during the late stages of infection where EEF size decreased threefold. Furthermore, electron microscopy data suggests a close interaction between host vesicular structures and the parasite Parasitophorous Vacuolar Membrane (PVM), suggesting that

these structures could be fusing with the PVM and be an important source of nutrients during parasite growth.

Taken together, these results point toward a novel role for host vesicles during *Plasmodium* late liver infection, possibly as source of nutrients and lipids for parasite growth. siRNA depletion also revealed a novel host-parasite interaction, with Rab1a, therefore offering new insights into the processes underlying *Plasmodium*-host interaction during the liver stage of infection.

Resumo

O agente causador da malária, o parasita Plasmodium, após ser transmitido pela picada de um mosquito, tem de invadir o mamífero hospedeiro e migrar até ao fígado, onde irá infectar um hepatócito. Após migrar através de várias células no fígado, o parasita invade o hepatócito final, formando uma membrana à sua volta, a membrana vacuolar parasitária (MVP). Nesta fase occorrem alterações morfológicas, bem como modificações na expressão génica, sendo que o parasita alcança uma das maiores taxas de replicação conhecidas nas de espécies eucarióticas (Sinnis 1997).

Sabe-se pouco sobre a formação e composição da membrana vacuolar parasitária (MVP), contudo à medida que o parasita matura e replica, este requer grandes quantidades de nutrients e lípidos. Neste sentido, as proteínas e lípidos do hospedeiro podem ser uma fonte importante de nutrients para este crescimento, estando a MVP no centro desta possível troca entre a célula hospedeira e o parasita.

Muitos patogénios intracelulares, para sobreviver, conseguem subverter as vias endocíticas e fagocíticas do hospedeiro estabelecendo um nicho intracelular único no qual sobrevivem e replicam. Usando técnicas de immunoflurescência, foi possível observar que o esporozoíto de Plasmodium, em hepatócitos, encontra-se adjacente ao retículo endoplasmático (RE) e ao complexo de Golgi da célula hospedeira. Peroxissomas e endossomas primários não se agregam em redor do parasita enquanto que endossomas tardíos e/ou lisossomas rodeiam o parasita durante toda a infecção no fígado.

Para estudar a função dos endossomas tardíos/lisossomas que se agregam em torno de parasitas *Plasmodium*, durante a infecção da malária, foi utilizada uma biblioteca de siRNA dirigida à família de Rab GTPases, proteínas que controlam o tráfico membranar. Apenas um tratamento siRNA teve um efeito significativo sobre a taxa de infecção parasitária no fígado sendo imprevisívelmente, Rab1a.

O papel da Rab1a foi caracterizado e seu efeito durante a infecção do Plasmodium no fígado foi investigada. A proteína Rab1a tem sido implicada no tráfico de RE-a-Golgi e, mais recentemente, no processo de autofagia, mais especificamente na formação de autofagosomas. Autofagia é um processo pelo qual o material citoplasmático é degradado e reciclado, tendo também uma função importante na destruição e eliminação antibacteriana, e deste modo vários patógenos desenvolveram mecanismos para evitar eliminação autofágica.

Inesperadamente, Rab1a parece estar a afectar o parasita Plasmodium através do seu papel durante a autofagia, e não pela via do tráfico retíulo endoplasmoático RE-a-Golgi. Rab1a parece ser importante para a morte de parasitas durante a fase inicial do seu desenvolvimento no hepatócito, uma vez que a sua ausência leva a um aumento na taxa de infecção parasitária. No entanto, durante os últimos estágios da infecção, Rab1a parece ser importante para a replicação do parasita, uma vez que parasitas às 40 horas após a infecção são menores nas células em que Rab1a foi silenciado. Estudos de colocalização usando LC3, como marcador autofágico, mostraram que as vesículas positivas para LC3 agregam-se em redor do parasita, sugerindo uma nova e importante função para a autofagia durante a infecção pelo *Plasmodium* no fígado.

A fim de melhor caracterizar a identidade das vesículas que se agregam em torno de parasitas *Plasmodium*, corantes sensíveis ao pH interno de vesículas intracelulares foram usados para mostrar que estas vesículas são ácidas, embora o espaço entre o parasita e a MVP se mantenha com um pH neutro. Surpreendentemente, vesículas positivas para Catepsina D, uma enzima hidrolítica presente nos lisossomas degradativos, não foram encontradas à volta do parasita. Para estudar o efeito destas vesículas ácidicas, células infectadas foram tratadas com cloreto de amónio, um tratamento que aumenta o pH no interior das células, inibindo o tráfico e maturação dos endossomas tardíos, corpos multivesiculares (CMV), anfissomas e lisossomas. Este tratamento não teve qualquer efeito sobre a taxa de infecção parasitária, mas prejudicou gravemente o crescimento do parasita, especialmente durante os últimos

estadios da infecção, em que o tamanho dos parasitas diminuiu significativamente. Um resultado semelhante foi obtido com concanamicina A, uma droga que actua como um inibidor da vATPase. Adicionalmente, dados obtidos usando microscopia eletrónica sugerem uma interacção próxima entre as vesiculas do hospedeiro e a MVP do parasita, propondo que aquelas estruturas podem estar a fundir com o MVP e ser uma fonte importante de nutrientes durante o crescimento do parasita.

Os resultados apresentados nesta tese identificam uma nova função para vesículas do sistema autofágico/endocítico do hospedeiro, possivelmente de anfissomas, durante a infecção pelo *Plasmodium*, mais especificamente, como uma possível fonte de nutrientes e lipídos para o crescimento do parasita. Os resultados também revelam uma proteína do hospedeiro, Rab1a, importante para o desenvolvimento do parasita dentro de células hepáticas, oferecendo assim uma nova perspectiva acerca dos processos que decorrem durante a fase hepática de uma infecção de malária.

Table of contents

Contents

1. **General Introduction**

1.1 History of malaria and importance in the history of humanity

The first known descriptions of malaria relate to a Chinese document from circa 2700 BC, a clay tablet from the Mesopotamia from 2000 BC, an Egyptian papyri from 1570 BC and Hindu texts from before the sixth century BC. Later Greek texts, showed that educated people were well aware of the typical symptoms associated with malaria, the fevers and the enlarged spleens, of people living around marshy areas. Interestingly the word malaria comes from the Italian "mala aria" meaning "bad air" (Bruce-Chuvatt, 1981). Although the ethological agent was still unknown, Hippocrates, in about 400 BC, describes the occurrence of intermittent fevers which occur daily or every-other-day in malaria patients as opposed to other infectious diseases.

After the discovery of bacteria by Antoni van Leeuwenhoek in 1676 and the discovery of microorganisms as causative agents of diseases, the search for the cause of malaria intensified. The first parasite was observed in the blood of patients by Charles Louis Alphonse Laveran in 1880, who observed several different forms of erythrocytic organisms inside and outside of red blood cells. He also described clear spots that grew, acquired pigment and filled red blood cells before bursting, coinciding with the fevers associated with malaria. Importantly, he noted that quinine removed these stages from the blood. He initially named this parasitic protozoan *Oscillaria malariae*. Although his theories were not well accepted at first, he was later awarded the Nobel Prize for Medicine in 1907, *"in recognition of his work on the role played by protozoa in causing diseases in humans"* (ww.nobelprize.org).

While there was a huge race in malaria research at this time, the entire life cycle was only described in birds by Manson and Ronald Ross in 1897, and subsequently in humans in 1899. Ross later won the Nobel Prize for Medicine in 1902 for this discovery. At the same time, a group of Italian scientists also reached the same conclusion, and noted that it was only female *Anopheles* mosquitoes that could transmit malaria, which quickly led to new measures to prevent malaria transmission by reducing contact with infected mosquitoes.

Although most of the life cycle was elucidated before the 1900s, nobody knew where the parasites developed during the first 7 to 10 days after infection, as they were not detected in the blood of patients. The elusive liver stage of infection was only discovered almost 50 years later by Henry Shortt and Cyril Garnham in 1948 (Shortt and Garnham, 1948). It was only in 1982, that the dormant exo-erythrocytic stages, the hypnozoites, were discovered by Wojciech Krotoski and colleagues, which elucidated the final stage of the malaria parasite life cycle in humans (Krotoski et al., 1982).

1.2 Malaria endemicity and burden in the world

Although the widespread implementation of intervention programs has diminished the burden of malaria in most countries where it was endemic, malaria is still one of the world´s leading causes of death. According to the World Health Organization, around 3.3 billion people – around half the world population- are at risk of malaria, and there are over 250 million new cases per year. Around 108 countries are endemic for malaria and nearly one million deaths occur every year due to this disease, the majority of which are African children under 5 years of age. (WHO Malaria Report 2009).

A recent review of malaria burden and the efficacy of intervention programs revealed that in most places, such as Southern Africa and the Horn of Africa, malaria burden has steadily and significantly decreased as intervention became more widespread and efficacy improved (O'Meara et al., 2010). Substantial increase in funding and an improvement in the procurement and distribution of effective means of prevention have been identified as the major causes for this decline. Nevertheless, in other parts of the world, such as in Central Africa, little progress has been documented (O'Meara et al., 2010).

Some of the key reasons for a steady decline in malaria prevalence have been the widespread change in drug treatment, from chloroquine (to which the parasite has now developed resistance to), to a cocktail of

sulphadoxine plus pyrimethamine or an artemisinin combination, and the scale-up of insecticide-treated bed nets and indoor residual spraying (O'Meara et al., 2010).

Alongside the human fatalities, the burden of malaria also extends to a significant economic impact in endemic countries, as it has been estimated that an average loss of 1.3% of annual economic growth is due to this disease (WHO Malaria Report 2009). This affects poor and rural areas more dramatically, where treatment is often lacking or scarce and where the population have limited access to healthcare. Pregnant women are at special risk of dying from complications of severe malaria and are also at high risk of spontaneous abortion, premature delivery and low infant birth weight. It is estimated that nearly 10 000 pregnant women and up to 200 000 infants die of malaria each year in Africa alone (WHO Malaria Report 2009).

For the past 50 years, huge efforts have been made to develop and produce a malaria vaccine although with little success. Early studies in the 1940s using irradiation attenuated parasites showed that these could indeed induce useful immunity to further infections in mice and in humans (Nussenzweig et al., 1967)(Clyde et al., 1973).

Two alternative approaches have been widely developed for human trials. These are immunization with intact attenuated liver stage parasites (sporozoites), which has shown various production problems when trying to scale up sporozoite production, and immunization with “sub-unit” vaccines based on immunogenic components of the sporozoite, the most promising of which is the RTS,S, that includes a polypeptide corresponding to the amino acids 207 to 395 of the Circumsporozoite Surface protein (CSP) from the human malaria parasite, *P.falciparum* (Stoute et al., 1997). The latter, has been recently tested on infants in a highly endemic area in Mozambique and shown to have a vaccine efficacy of approximately 66%, a promising result although more testing is required (Aponte et al., 2007)(see (Vanderberg, 2009) for a very good review of the history of vaccine development by one of its initial founding co-investigators).

1.3 Overview of Plasmodium life cycle

During its life cycle, *Plasmodium* parasites take many forms and shapes. As the last of these stages was only discovered around 50 years ago, with the discovery of the pre-erythrocytic liver stage and the hypnozoite stage, a clear and complete image of the entire life cycle in mammals was only achieved in the second half of the last century.

In all *Plasmodium* species, the complete life cycle requires two different hosts, mammalian and insect, more specifically a female *Anopheles* mosquito. The mammalian life cycle initiates when an infected mosquito is looking for a blood meal and probes for a blood source under the dermis of a mammalian host. The sporozoites, the name given to this stage of the parasite life cycle, are injected during salivation and migrate through the dermis in order to find a blood vessel in which to travel to the host´s liver. Once in the liver, sporozoites need to transverse the liver sinousoid and reach hepatocytes, migrating through several hepatocytes before committing to infection (Mota et al., 2001)(Mota et al., 2002). Inside the final hepatocyte sporozoites invade this cell, with the formation of a Parasitophorous Vacuole Membrane (PVM) and initiate the liver stage of infection. Here the parasites, often termed exo-erythrocytic forms (EEFs), undergo distinct morphological changes and replicate for around 2 to 16 days, depending on the *Plasmodium* species. This stage is often called the silent stage of infection as it is asymptomatic. Once the parasites have fully replicated, producing around 10,000 to 30,000 blood stage parasites (merozoites), these are released into the vasculature (Prudêncio et al., 2006). Here, free merozoites are able to invade red blood cells (RBCs) and undergo another replication cycle, producing 16 to 32 new merozoites which rupture and infect new RBCs.

This synchronous blood stage infection coincide with the cyclic fevers associated with malaria and can lead to death if left untreated. Malaria

symptoms include rupture of the blood-brain barrier inducing coma and other neurological abnormalities and eventually death (also called “severe malaria”), as well as other milder symptoms such as fevers, headache, vomiting, metabolic acidosis which can lead to respiratory distress, severe anemia and multi-organ failure. Also during the blood stage some merozoites develop into sexual stage parasites, the male and female gametocyte, and, if taken up by another female mosquito, the life cycle can continue in the insect host (**Figure 1.1**).

Very briefly, in the mosquito midgut, exflagelation of the microgametocytes (male) occurs and the marogametocyte (female) is fertilized. The resulting ookinete migrate to the mosquito hemocele and develop into an oocyst, where the sporozoites are formed. These then burst and migrate into the salivary glands where they become infective and ready to be transmitted to the next host. A more detailed description of the mammalian life cycle, the focus of this project, will be described below.

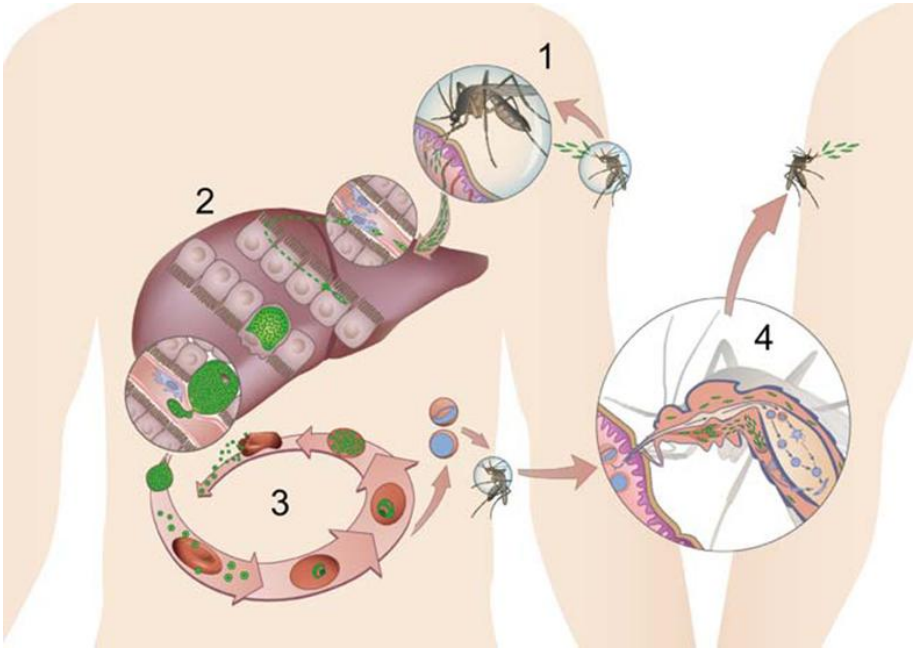


Figure 1.1 – Plasmodium life cycle in the human and mosquito host. (1) The parasite life cycle begins with the inoculation of sporozoites into the dermis of the mammalian host by an infected female *Anopheles* mosquito. (2) Sporozoites quickly reach the host blood stream and get to the liver, where they migrate through several hepatocytes before committing to infection, with the formation of the Parasitophorous Vacuole membrane (PVM). Here sporozoites grow and multiply, developing into large liver schizonts. (3) Liver schizonts finally develop into thousands of merozoites which are released into the blood stream, initiating the blood stage of infection and the subsequent infection of red blood cells. Some parasites will eventually develop into sexual gametocytes which can be taken up by a mosquito during a new blood meal. (4) Once in the mosquito midgut, parasites undergo a series of transformations which culminate in the development of new infectious sporozoites in the mosquito´s salivary glands, which can infect a new mammalian host. Image adapted from Prudêncio et al, 2006.

1.4 The mammalian life cycle in more detail

1.4.1 Insect biting and the "dermis stage"

Malaria transmission initiates when an infected female Anopheles mosquito probes for a new blood meal on a mammalian dermis. The sporozoites, which have developed and reached the salivary glands of the mosquito, are deposited into the skin during salivation along with

anticoagulants present in the saliva of the mosquito (Griffiths and Gordon, 1952) This stage of infection, recently termed the "dermis stage", was previously thought to have no significance in the infection process although a large body of evidence has showed that the hosts immune response to sporozoites actually begins to be mounted in the skin (Sinnis and Zavala, 2008).

The number of sporozoites deposited in the dermis in one blood meal varies between different studies but between 100 to 200 sporozoites are believed to be deposited in the skin during one blood meal (Frischknecht et al., 2004)(Medica and Sinnis, 2005)(Jin et al., 2007). Although they can remain in the dermis for several hours (Yamauchi et al., 2007), most sporozoites quickly migrate through the avascular dermis in order to find a blood vessel and reach the circulatory system (Vanderberg, 1974)(Vanderberg and Frevert, 2004). Migration, a process where a parasite ruptures the cell membrane, moves through the cell cytoplasm and comes out the other end, is achieved due to a specialized and complex molecular apparatus powered by a unique subpellicular actomyosin motor that is linked to the sporozoite surface and is common to all Apicomplexa parasites (Keeley and Soldati, 2004).

Once in the dermis of the host, a parasite has one of three possible fates; close to 50% stay in the dermis. From the remaining 50%, 70% are able to invade a blood vessel whilst the remaining 30% invade a lymphatic vessel and reach a lymph node (Amino et al., 2006). Only if a parasite is able to migrate, find a blood vessel, be transported to the liver and cross the liver sinusoid, will an active infection ever occur.

The "dermis" stage of infection has only recently been studied revealing the interesting findings that sporozoites injected into immunized mice are rapidly immobilized in the skin, did not appear to reach blood vessels and died in the skin within several hours (Kebaier et al., 2009). These results indicate that protective antibodies against the sporozoite in the skin may help in reducing further infection by blocking their movement into blood vessels and

ultimately the liver, which could aid in mounting immunity to pre-erythrocytic stages and form a basis for malaria vaccine design (Sinnis and Zavala, 2008).

Various parasite proteins essential for migration and gliding motility, both in the dermis and later in the liver, have been described (see **Figure 1.2**). The Thrombospondin-Related Anonymous Protein (TRAP) and the Apical Membrane Antigen 1 (AMA1), are two parasite proteins which are up-regulated during the sporozoite stage (Rogers et al., 1992b)(Rogers et al., 1992a)(Robson et al., 1997)(Silvie et al., 2004). TRAP is the primary motor-binding protein of sporozoites (Sultan et al., 1997), while AMA1 has been shown to be part of the structural component of the moving junction (Mitchell et al., 2004).

The Circumsporozoite Protein (CSP), the major sporozoite surface protein, is also essential for parasite migration not only in the mammalian but also within the mosquito host (Ménard et al., 1997)(Myung et al., 2004), but its major role in the parasite life cycle is during liver development, as will be described later.

Various parasite proteins involved in cell transversal have also been identified. The Sporozoite Microneme Protein Essential for Cell Transversal (SPECT2), the Cell Transversal Protein for OOkinetes and Sporozoites (CelTOS) and Phospholipase (PL) have all been described as parasite proteins essential for cell transversal activity (Ishino et al., 2004)(Ishino et al., 2005a)(Bhanot et al., 2005)(Kariu et al., 2006). (Other proteins involved with migration can be reviewed in Vaughan et al 2008.)

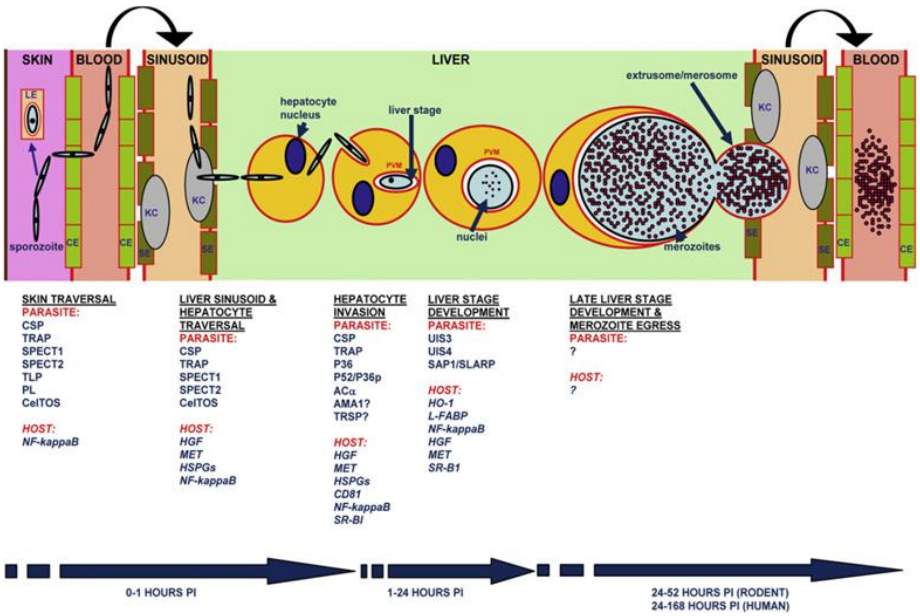


Figure 1.2 – Molecules involved in Plasmodium invasion and development in the mammalian host. The various stages of parasite development inside the mammalian host are depicted. Both parasite and host proteins known to be important for each stage of development are listed. CE, endothelial cells; LE, lymphoid endothelium; SE, fenestrated endothelia; KC, Kupffer cell; PVM, Parasitophorous vacuole membrane; PI, post infection. Image adapted from Vaughan et al, 2008.

1.4.2 Arrest and attachment to hepatocytes

Once in the host circulatory system, sporozoites may be found in hepatocytes within 2 minutes of intravenous injection into rats (Shin et al., 1982). This rapid arrest in the liver indicates that a very strong and specific interaction must occur between the parasite surface proteins and the hepatocyte surface proteins, although relatively little is known about this process.

A large body of evidence has shown that the major parasite surface protein, the Circumsporozoite protein (CSP), is able to interact directly with the heparin sulphate proteoglycans (HSPGs) on liver cells. The sinusoid is composed mainly of endothelial cells and Kupffer cell, which have open fenestrations allowing for small lipoproteins, such as HSPGs to extend into the lumen of the blood vessels, to the space of Disse, and directly interact with

CSP from circulating parasites (Sinnis et al., 1996). Although HSPGs are present in most tissues in the mammalian body, liver HSPGs are known to be more highly sulphated than in all other tissues (Lyon et al., 1994). This feature has been proposed to be responsible for the selective binding of sporozoites to the liver although more research is required to confirm this (Ying et al., 1997)(Pinzon-Ortiz et al., 2001).

More recently, another parasite protein, TRAP, has been shown to be involved in this sequestration in the liver (Pradel et al., 2002)(Pradel et al., 2004). In addition, liver stellate cells are able to synthesize eight times more sulphated HSPGs than hepatocytes and incorporate twice the amount of sulphate into heparan sulphate (Gressner and Schäfer, 1989). Thus, it has been suggested that parasite arrest in the liver may be mediated by attachment to a matrix of HSPGs that protrude from the endothelial fenestrations and are produced by a series of cells in the liver in very high amounts (Pradel et al., 2002)(Pradel et al., 2004).

In order to reach hepatocytes, sporozoites have to transverse the sinusoidal liver barrier, composed mainly of endothelial and Kupffer cells. The importance of and route by which parasite are able to transverse through either of these cells has been a topic of some controversy. Endothelial cells have fenestrations but these are too small for parasite to pass through (about one tenth of the diameter of a parasite).

Strong evidence suggests that parasites cross the sinusoidal layer primarily through Kupffer cells and that these cells provide a doorway into hepatocytes (Baer et al., 2007). Kupffer cell invasion with the formation of a PVM has been observed (Pradel and Frevert, 2001) and sporozoites are able to survive this cell passage unharmed by suppressing the reactive oxygen species by secreting cyclic adenosyl monophosphate (cAMP) (Usynin et al., 2007). Furthermore, experiments with the SPECT1 and SPECT2 deficient *P.berghei* sporozoites, which are defective in their ability to transverse cells, have a very low infectivity in vivo (Ishino et al., 2004)(Ishino et al., 2005b), suggesting that

migration through cells is an important step in establishing a successful infection (see (Frevert et al., 2006) for a good review on this topic).

The choice of hepatocytes as the preferred cell for the next stage of the *Plasmodium* life cycle is still a mystery. Nevertheless, compared to other cells, hepatocytes have unique metabolic properties, being particularly proficient at metabolizing compounds (e.g. lipids, purines, glucose) in large quantities (Morgan and Baker, 1986)(Pels Rijcken et al., 1993)(Klover and Mooney, 2004). The fast multiplication rate of *Plasmodium* parasites in the liver impose a high demand on lipids for organelle synthesis and for this reason hepatocytes represent a favorable environment within the mammalian host, especially for a parasite that is unable to synthesize sterols. Interestingly, infected cells seem to upregulate proteins involved in sterol synthesis and lipid metabolism (Albuquerque et al., 2009), suggesting that *Plasmodium* parasites are able to manipulate host transcription to their own advantage and survival.

Whatever the process to reach the liver hepatocytes may be, eventually, successful parasites reach the host hepatocytes where they will ultimately commit to infection. Yet, before this occurs, they migrate through a series of hepatocytes, breaching the plasma membrane and passing in direct contact with the host cytosol (Mota et al., 2001)(Mota and Rodriguez, 2004).

Sporozoite migration, first in the skin and later in the liver, seems to be important in priming the sporozoite to commit to infection. Contact with the host cytoplasm, with its elevated Ca^{2+} concentration, induces exocytosis from the sporozoite apical organelles, forming an apical "cap" of proteins which will be important for subsequent infection (Gantt et al., 2000)(Mota et al., 2002)(Kumar et al., 2007). In addition, host cell wounding in the liver by parasite migration induces the secretion of hepatocyte growth factor (HGF), rendering neighboring hepatocytes more susceptible to infection (Carrolo et al., 2003) and protects them from apoptosis (Leirião et al., 2005).

1.4.3 Liver stage migration, invasion and PVM formation

Once sporozoites have migrated through a series of hepatocytes, they are ready to invade, a process is actively controlled by the parasite, using its glideosome (motility) machinery to force its way into the host cell (Keeley and Soldati, 2004). Invasion occurs by a partial invagination process of the host cell plasma membrane (Mota et al., 2001). Specific parasite adhesins are secreted at the parasite apical end and are proteolytically processed. Processing of these proteins is believed to expose the adhesive domains which function at the sporozoite-host cell interface and help the sporozoite to adhere to the hepatocyte plasma membrane (reviewed in (Carruthers and Blackman, 2005)).

Apart from proteins involved in parasite gliding motility, only a limited number of parasite and host proteins have been shown to be involved in parasite invasion (Vaughan et al., 2008)(Ejigiri and Sinnis, 2009). *P.berghei* Pbs36p and Pbs36 proteins are members of a family of proteins with 6 conserved cysteine residues which are specifically produced in liver-infective sporozoites. When expression of these proteins is abolished, infectivity in the mammalian host is inhibited, although cell transversal is not affected (Ishino et al., 2005b). This result suggests that these proteins are necessary for sporozoites to recognize hepatocytes and commit to infection.

Two host hepatocyte proteins, scavenger receptor SR-B1 and the tetraspanin CD81, have recently been shown to have a role in parasite invasion. SR-B1 is a major provider of cholesterol to the hepatocyte, a nutrient which seems to be critical to parasite growth (Leirião et al., 2005). SR-B1 transgenic mice, which express only approximately 10% of normal protein level, were less permissive to sporozoite infection while transgenic mice where SR-B1 expression was increased had an enhanced permissiveness to both *P.berghei* and *P.yoelii* infection (Yalaoui et al., 2008a). The same occurred when human hepatoma cells (HUH7) were treated with siRNA reducing SR-B1 expression (Rodrigues et al., 2008).

CD81 is a tetraspanin protein that associates with other proteins and lipids to form membrane microdomains. CD81 deficient mice were also less permissive to *P.yoelii* sporozoite invasion (Silvie et al., 2003) and later studies found that cholesterol was involved in the assembly of CD81 microdomains (Silvie et al., 2006). Since SR-B1 is a provider of hepatocyte cholesterol, it has been hypothesized that this protein plays a role in the formation of CD81 enriched microdomains which in turn facilitate sporozoite entry (Yalaoui et al., 2008b). SR-B1 deficient mice showed a decreased expression of CD81 on the cell surface indicating that some membrane rearrangement event had occurred, which in turn had an effect on parasite invasion. Further studies are needed to confirm all of these hypotheses.

As mentioned previously, sporozoite invasion is characterized by the formation of a PVM, yet the sequence of events leading to PVM formation are largely unknown. Three different mechanisms of PVM formation have been proposed: 1) de novo PV membrane formation solely from secreted parasite proteins, 2) PVM formation from direct invagination of the host plasma membrane or 3) a combination of both mechanisms (reviewed in (Mota et al., 2002)). Further studies are required to unravel this unique process.

1.4.4 Development within the hepatocyte

Once inside the host hepatocyte, sporozoites undergo one of the most rapid replication rates in the eukaryotic world (Sinnis and Sim, 1997). It has been estimated that from one single sporozoite, around 10 000 to 30 000 merozoites are formed after 3-14 days, depending on the *Plasmodium* species (Prudêncio et al., 2006). Such a rapid replication rate implies that various host-parasite interactions must occur in order to supply the parasite with necessary nutrients, thus various parasite proteins as well as host proteins must be involved in such dynamic interactions. These interactions primarily occur at the PVM, a deeply convoluted membrane, that is permeable to small molecules

and ions (Bano et al., 2007) yet its composition and origin is still largely unknown.

Within the liver, sporozoites differentiate into exo-erythrocytic forms (EEFs), that grow and multiply (schizont stage) forming thousands of new erythrocyte infectious merozoites (**Figure 1.3**) (Sturm et al., 2009). These newly formed merozoites are subsequently surrounded by a membrane resulting in fully mature merozoites which are ready to be released into the liver blood vessels.

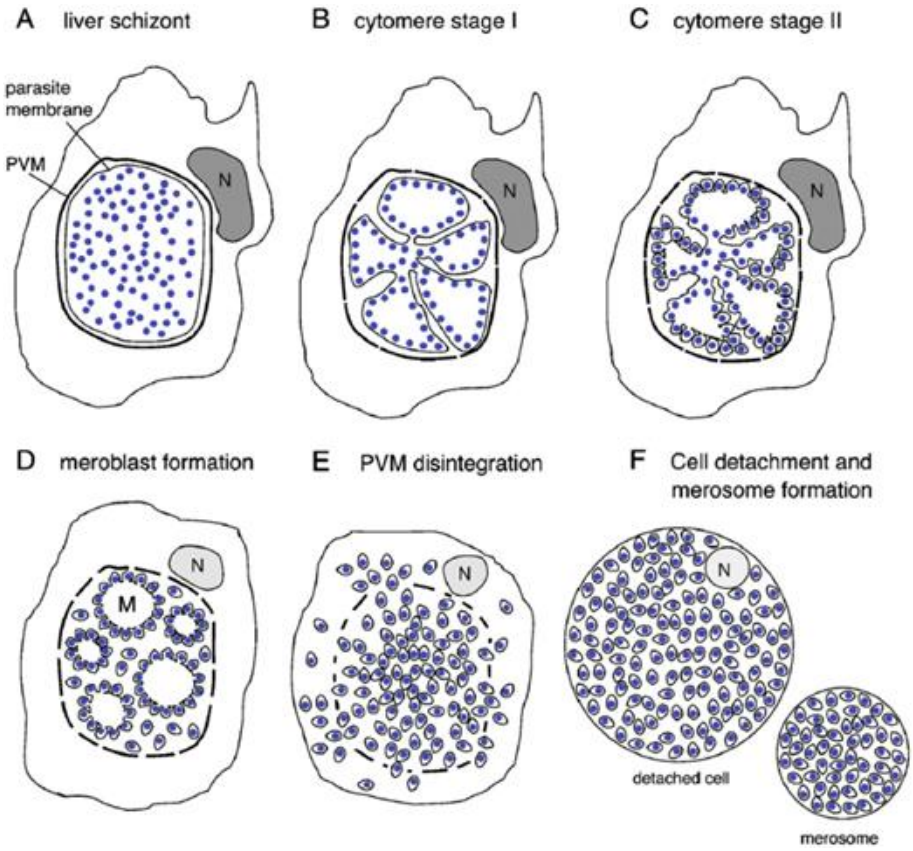


Figure 1.3 – Model for late liver stage development. (A-F) Schematic representation of late liver stage development, where late liver schizont´s PVM undergoes invagination (A-B), surrounding large groups of newly formed parasite nuclei, until single nuclei are fully membrane bound (C), forming merozoites within a meroblast (D). The PVM disintegrates (E), resulting in merozoite release into the host cytoplasma, followed by detachment of merozoite filled vesicles called merosomes into blood vessels (F). PVM, Parasitophorous Vacuole Membrane; N, nucleus. Image adapted from Sturm et al, 2009.

Largely due to the technical difficulties involved in studying this stage of the life cycle, to date, only a few parasite proteins have been shown to play a role in parasite liver development. Salivary gland sporozoites upregulate the expression of a unique subset of genes collectively called the UIS (upregulated in infectious sporozoites) and some of these have been shown to be essential for liver stage development (Matuschewski et al., 2002)(Mikolajczak et al., 2008). More recently it was shown that a conserved *Plasmodium* sporozoite low-complexity asparagine-rich protein, SAP1 (Sporozoite Asparagine-rich Protein 1) is involved in a selective post-transcriptional mechanisms to regulate the abundance of UIS transcripts (Aly et al., 2008)(Aly et al., 2011).

Up-regulated in Infective Sporozoite genes 3 and 4 (UIS3 and UIS4) are two transmembrane proteins that localize to the PVM just a few minutes after invasion. If disrupted, sporozoites are unable to establish an adequate liver infection, as knock out parasites invade hepatocytes normally but dies within the first 48 hours of infection (Mueller et al., 2005). These parasites demonstrate the importance of the PVM during the liver stage of infection. It is known that UIS3 binds directly to liver fatty acid binding protein (L-FABP) on the PVM (Mikolajczak et al., 2007)(Sharma et al., 2008) while there is still no known function for UIS4.

A significant amount of lipids are required to ensure the generation of thousands of new merozoites. In order to achieve this parasites have evolved a type II fatty acid synthesis (FASII) pathway, distinct from the type I found in mammals. Disruption of parasite enzymes essential in this pathway, FabB/F, FabZ and Fab1, render parasites unable to develop in the liver and form merozoites (Yu et al., 2008)(Vaughan et al., 2009). Thus, it seems that parasites are able to not only synthesize their own fatty acid requirements, which are essential for their development, but also sequester fatty acids from the host cells (Albuquerque et al., 2009). These two mechanisms are both required for proper parasite development and must interact in some as yet uncharacterized

manner, as down-regulation of host L-FABP severely impairs liver development (Vaughan et al., 2009).

P36p, another parasite protein, has been shown to be important during invasion, development and PVM formation although there is conflicting evidence on its exact role (van Dijk et al., 2005)(Ishino et al., 2005a)(Labaied et al., 2007)(Ejigiri and Sinnis, 2009).

Even if many of the molecular functions of some of these proteins is not yet known, their importance in liver stage research has been immeasurable, as parasites mutated for these proteins, don´t develop fully in the liver yet illicit protective immunity to further sporozoite challenges, thus being termed genetically attenuated sporozoites (GAS). In a similar way to radiation attenuated sporozoites (RAS), this model has allowed for a deeper understanding of the basic immune mechanisms that mediate sterile immunity against reinfections (Nussenzweig et al., 1967)(Hafalla et al., 2006).

During its liver development, the parasite largely outgrows the normal host cell limits and is able to remodel and exploit the host hepatocyte (reviewed by (Mikolajczak and Kappe, 2006)). Nevertheless, very few host proteins have been implicated in this process.

Studies performed investigating the roles of the parasite UIS4 have shown that this protein colocalizes with host ApoA1 in the PVM 24 hours after parasite invasion and ApoA1 has been speculated to be important in the synthesis of large amounts of membranes essential for the PVM expansion (reviewed in (Prudêncio et al., 2006)).

UIS3 was also shown to interact directly with L-FABP using a two-hybrid system (Mikolajczak et al., 2007). L-FABP down regulation in hepatocytes severely impairs parasite growth and its overexpression promotes parasite growth. L-FABP has been speculated to be the main carrier of fatty acids from the host cell cytoplasm to the parasite yet further studies are required in order to confirm its role in parasite development.

Host Hepatocyte Growth Factor (HGF) is secreted by hepatocytes during parasite migration and has been shown to induce host actin cytoskeleton

rearrangements via the HGF receptor, MET, signaling pathway (Carrolo et al., 2003). It was also shown that this signaling cascade prevents infected cells from undergoing cell death by apoptosis, a mechanism that would be very detrimental for the parasite (Leirião et al., 2005). Curiously, infected hepatocytes do not display the normal signs of cell death and were actually resistant to tumor necrosis factor ((TNF)-α/D-galactosamine) stimulation (van de Sand et al., 2005), showing that the parasite is able to constantly stimulate host cell survival throughout the duration of its liver stage infection (reviewed in (Heussler et al., 2006)).

Apart from its role in parasite invasion, host SR-B1, also seems to play a role in parasite development, as parasite in SR-B1 transgenic mice had a diameter 3-fold larger than in SR-B1 knock-out mice at 48 hour post liver infection (Yalaoui et al., 2008a). Interestingly, SR-B1 was not localized around the PVM which has led to the hypothesis that SR-BI effects parasite development due to its interaction with L-FABP, although more evidence is required to confirm this hypothesis (Rodrigues et al., 2008)(Yalaoui et al., 2008b).

Taken together, these studies show that *Plasmodium* parasites are able to actively manipulate and hijack a wide range of host cell functions and pathways, in order to establish its own specialized intracellular niche. Host cell subversion is a common strategy used by intracellular pathogens to ensure survival and adequate growth. To achieve this pathogens utilize a wide range of techniques and secrete a multitude of proteins or effectors. Unfortunately, due to the difficulties involved in *Plasmodium* liver stage research, no such proteins have been identified for this intracellular pathogen.

By comparison with the Parasitophorous vacuole of other Apicomplexa parasites which associate with diverse host organelles, the *Plasmodium* Parasitophorous vacuole has only been shown to associate with the host endoplasmic reticulum at 24 hours post infection (Bano et al., 2007). Intrahepatic *Plasmodium* actively modifies the permeability of its vacuole to allow the transfer of a large variety of molecules from the host cytosol to the

vacuolar space through open channels (Sturm et al., 2009)(Bano et al., 2007). In contrast with malaria blood stages, the pores within the Parasitophorous vacuole membrane of the liver stage display a smaller size as they restrict the passage of solutes to less than 855Da. These pores are stably maintained during parasite karyokinesis until complete cellularisation. Host-derived cholesterol accumulated at the Parasitophorous vacuole membrane may modulate the channel activity (Labaied et al., 2010). These observations describe the Parasitophorous vacuole of the *Plasmodium* liver stage as a dynamic and highly permeable compartment that can ensure the sustained supply of host molecules to support parasite growth in the nutrient-rich environment of liver cells (Bano et al., 2007). We were thus interested in exploring this in more detail.

1.4.5 Leaving the liver and into the blood

After parasites have developed in the liver, the pre-erythrocytic development comes to an end and the newly formed merozoites are ready to invade RBCs. It was initially believed that merozoites, after rupturing their PVM and coming into contact with the hepatocyte cytoplasm, were released into the blood stream by a rupture of the host cell membrane, although this did not explain how the merozoites were able to cross the extracellular matrix and pass through the endothelium sinusoid layer. Early studies found merozoite filled membrane bound vesicles within the extracellular matrix (Meis and Verhave, 1988). This membrane was later found to be of host origin, which explains why these vesicles, termed merosomes, are not attacked by phagocytic cells in the liver, and are able to pass unharmed through the sinusoidal space and into the blood vessels (Sturm et al., 2006) (**Figure 1.4**).

A more recent study showed that merosomes remain intact after their passage through the liver sinusoid, through the heart and become sequestered within the lung capillaries. Here the merosome membrane disintegrates and

liberates the fully formed merozoites into the lung circulation (Baer et al., 2007). This phase of the life-cycle is believed to enhance the dissemination of merozoites into the blood circulation where they will encounter red blood cells and initiate the next round of infection in the mammalian host.

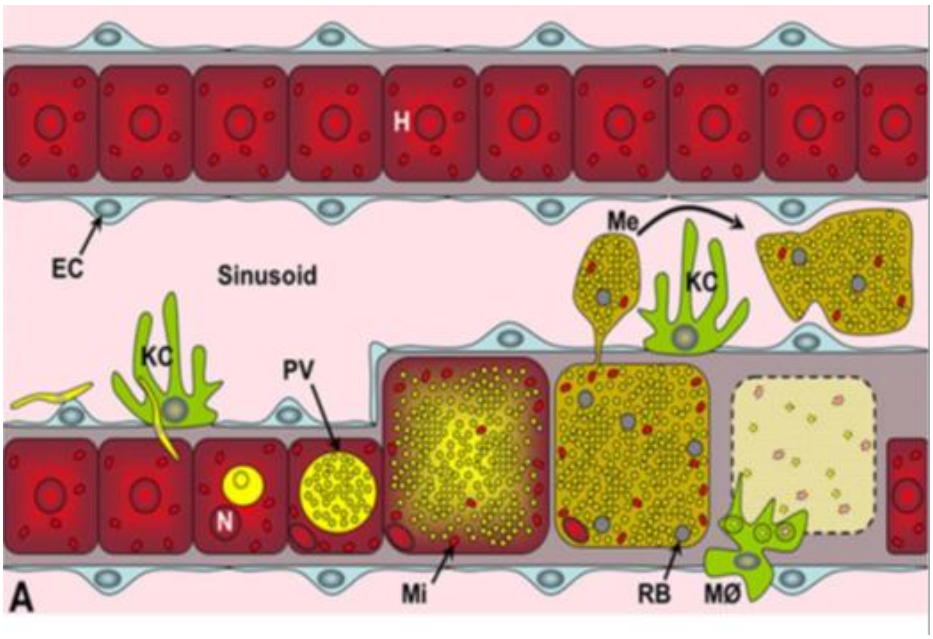


Figure 1.4 - Model for merosome dissemination and liberation into the blood. Model for merosome dissemination and liberation, surrounded by hepatocyte plasma membrane, avoiding recognition by Kupffer cells in the sinusoidal space; M, meroblast; H, hepatocyte; EC, endothelial cell; N, nucleus; KC, Kupffer cells; PV, Parasitophorous vacuole; Mi, host cell mitochondria; Me, merosomes; RB, remenant bodies; MØ, mononuclear phagocytes. Image adapted from Baer et al, 2007.

LISP1 (liver-specific protein 1) is a parasite protein known to be involved in the controlled release of merozoites into the blood stream. This protein is expressed during late liver stage development and is targeted to the PVM. LISP1- deficient liver stage parasites develop into mature, viable merozoites but are unable to rupture the PVM membrane, and so remain trapped inside the hepatocyte, showing that this protein is involved in the lysis of the PVM during late liver stage development (Ishino et al., 2009).

Exit from infected host cells also appears to be mediated by a class of papain-like cysteine proteases called Serine Repeat Antigens (SERAs). Members of this family, such as PbSERA1 and PbSERA2, are abundantly expressed in the final stages of merozoite formation and are targeted to the PVM. Single loss-of-function mutant parasites for PbSERA1 or PbSERA2 progressed normally throughout the parasite life cycle although the expression of other members of this family, namely PbSERA3 was upregulated, suggesting that members of this family can compensate for each other (Putrianti et al., 2010).

The next phase of the *Plasmodium* life cycle starts with the invasion of uninfected red blood cell (RBC) in the blood. The erythrocytic development initiates with parasites attaching and repositioning themselves on the RBC membrane. Once inside, parasites develop into the ring stage (0-24h), followed by the trophozoite (24-36h) and finally the schizont stage (40-48h) (Maier et al., 2009). After the first round of RBC development, parasites are released into the blood stream and initiate the new cycle of RBC invasion, giving rise to all the malaria associated symptoms (**Figure 1.5**).

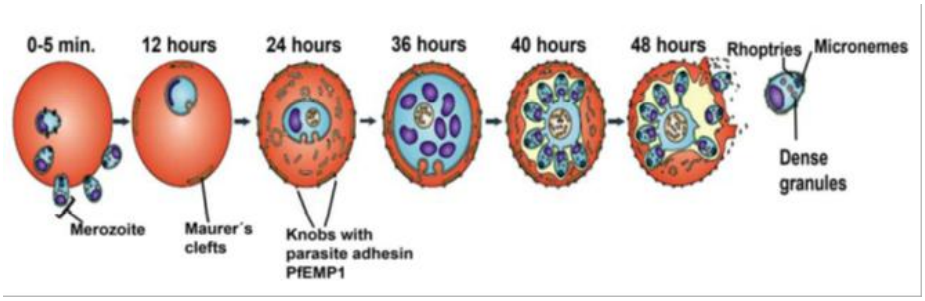


Figure 1.5 – Model for red blood cell invasion and development. *Plasmodium* merozoites attach and reorient on the surface of red blood cells (RBCs). Invasion occurs, with the formation of a vacuole membrane. A series of developmental transformations occur until finally, mature merozoites are released after replication cycles of 48 hours. Image adapted from Maier et al, 2009.

1.5 Models for liver stage infection

One of the most important breakthroughs in malaria research was the development of *in vitro* blood culture systems, pioneered by William Trager and J.B. Jensen in 1976 (Trager and Jensen, 2005). This opened the path to vaccine and drug development research. Yet, models for liver stage development were more difficult to develop. The liver stage is still the most elusive stage of the entire life cycle of the *Plasmodium* parasites. Although it was identified more than 50 years ago (Shortt and Garnham, 1948), technical constrains has made it very difficult to study the molecular mechanisms involved in this stage of infection. Human liver stage research is not feasible in vivo, although some studies have been able to mimic the complete liver stage development in human primary hepatocytes (Mazier et al., 1984)(Mazier et al., 1985)(Mazier et al., 1987), yet these experiments rely on the often scarce and unpredictable availability of human material, making this type of experiments very technically challenging. Complete *Plasmodium falciparum* and *P vivax* liver stage development has been achieved in human hepatocyte cell lines, HUH7 and HepG2-A16 respectively (Uni et al., 1985)(Calvo-Calle et al., 1994). More recently, the HC-04 cell line was able to support the complete liver stage development of both *P.falciparum* and *P.vivax* (Sattabongkot et al., 2006) although in all these experiments, mosquito breeding and infection with human parasites is required, all of which involve very specific and controlled safety protocols, thus making it very difficult and expensive to perform.

As a result of these challenges, several non-human models, including mouse models have been successfully developed to address the various stages of the *Plasmodium* life cycle (Langhorne et al., 2011). In particular, *Plasmodium berghei* and *P.yoelii* have been extensively used as well-suited model for *Plasmodium* liver stage development (Mota and Rodriguez, 2000)(Prudêncio et al., 2006). This has enabled a huge advance in this area of research in the past decades. Both *P.berghei* and *P.yoelii* are able to infect mouse primary hepatocytes as well as the mouse hepatoma cell line Hepa1-6

(Mota and Rodriguez, 2000). *P.berghei* is known to be more promiscuous than *P.yoelii* as it is also able to infect human hepatoma cell lines such as HepG2 and HUH7. More recently, a humanized mouse model was developed to study *P.falciparum* liver infection, providing a new tool to study this part of the life stage using this human pathogen (Morosan et al., 2006).

Both rodent models have different infection efficiencies, with *P.berghei* having the highest efficiency *in vitro* and *P.yoelii* having the highest efficiency *in vivo* (Khan and Vanderberg, 1991)(Briones et al., 1996). Most of the work in this project involved *in vitro* work and *P.berghei* parasites were used throughout.

Plasmodium berghei in vitro infection rates vary between 1-5% while *Plasmodium yeolli* infection rate are often even lower. This makes using cell biology and molecular biology techniques very difficult, which ultimately slowed down research in this area. Liver stage research also involves the growth and maintenance of live and infected *Anopheles* mosquitoes, a technique that is not easy to master. All these factors together, has made malaria liver stage research slow and difficult, even during the past 20 years where the number of publications in this area has skyrocketed.

1.6 Eukaryotic membrane traffic

A central question in cell biology is how proteins and other materials are distributed and transported among the intracellular compartments of a eukaryotic cell. A hallmark of eukaryotic cells is that they are highly compartmentalized and have evolved mechanisms to regulate intracellular transport, not only between intracellular compartments, but also to release material to the plasma membrane (secretion/ exocytosis) or to internalize material from the extracellular space, a process termed endocytosis (Bonifacino and Glick, 2004). Central to these transactions are proteins that interact with membranes, forming and reshaping them, to ensure that cargo is delivered to

the correct destination. It is the precise regulation between these different processes that ensures efficient intracellular transport while preserving correct organelle identity.

In order to transport cargo between organelles, vesicle budding or formation needs to occur from a "donor" compartment by a process that allows for the sorting of the received cargo. The vesicles are later targeted and fuse with a specific "acceptor" compartment (vesicle targeting and fusion). To ensure correct organelle homeostasis, these transport machinery components are recycled back from the acceptor compartment in a pathways termed "retrograde transport". **Figure 1.6** depicts the various intracellular transport pathways.

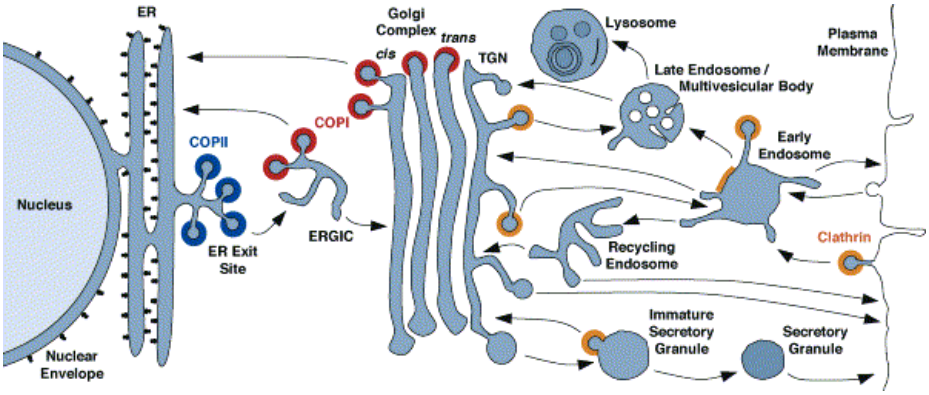


Figure 1.6 - Intracellular Transport Pathways. The three most important intracellular transport pathways are depicted in this scheme; the secretory or exocytic pathway, where cargo is delivered to the plasma membrane via secretory granules; the endocytic pathway, where cargo is internalized from the extracellular space; and the lysosomal/vacuolar pathway where cargo that has been internalized is processed to the cell interior. Adapted from Bonifacino and Glick, 2004.

All of these steps are tightly regulated by various families of proteins. Vesicle budding and cargo selection is mainly mediated by protein coats, such as Clathrins and Coat Protein Complex I and II (COPI and COPII) proteins (Pucadyil and Schmid, 2009) These proteins are able to self-assemble and deform membranes and directly interact with cargo molecules to ensure correct uptake. The GTPases Arf, Sar1 and dynamin are core components of the coat

vesicle machinery and can also actively remodel membranes (Scales et al., 2000).

Once formed, vesicle targeting and fusion is largely mediated by proteins termed the SNAREs (Soluble N-ethylmaleimide-sensitive factor activating protein receptor) (Gerst, 1999)(Duman and Forte, 2003). SNAREs have been extensively studied and mediate vesicle fusion by bridging two membranes (Ungar and Hughson, 2003). Individual SNARE family members tend to be compartment-specific and are thus thought to contribute to the specificity in docking and fusion events between organelles (Pelham, 2001) although it has been shown that some SNAREs can compensate for the loss of another or even act in multiple distinct complexes (reviewed in (Ungar and Hughson, 2003)).

Another family of proteins, the Rab GTPases, is also involved in multiple regulatory processes along these transport pathways as well as mediating the interaction of membranes with cytoskeletal motors. This family of proteins and their role in membrane traffic will be discussed further.

1.6.1 Rab GTPases as regulators of membrane traffic

A hallmark of eukaryotic cells is that they are highly compartmentalized and have evolved mechanisms to regulate intracellular transport between compartments. A number of intracellular pathogens are able to manipulate components of these mechanisms to aid their survival. Rab proteins, the largest branch of the Ras superfamily of small guanosine triphosphates (GTPases), are master regulators of organelle identity and play a key role in intracellular trafficking by controlling vesicle fusion and motility.

Eleven Rabs (Yptp/Sec4p) have been identified in Yeast while there are over 60 in some mammalian species (Stenmark and Olkkonen, 2001). This increase in complexity throughout evolution, due to duplications and adaptation

to specific cell types, reflects the great complexity and organization required in higher eukaryotic organisms (Pereira-Leal and Seabra, 2001).

Rab proteins function as molecular switches, cycling between their active GTP-bound state and their GDP-bound inactive state. As Rabs do not have high intrinsic guanine nucleotide exchange or hydrolysis activities, they require the recruitment of guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs) respectively to switch between their active and inactive forms (Pfeffer and Aivazian, 2004). Once in the active GTP-bound form Rab proteins can recruit a diverse array of effector molecules such as tethering factors, molecular motors and enzymes to perform their downstream biological activity. It is the coordinated spatial and temporal control between each Rab, its effector and the recruitment of specific GEFs and GAPs that gives Rabs the ability to tightly and specifically regulate intracellular membrane traffic (Barr and Lambright, 2010) (see **Figure 1.7**).

In their inactive GDP-bound state, Rab proteins are largely retained in the cytoplasm bound to Rab guanine nucleotide dissociation inhibitor (GDI). Once they have been recruited to their target membrane, the Rab-GDI complex interacts with GDI displacement factors which disrupts the Rab GDI complex and allows the Rab to be inserted into the membrane. Concomitant with membrane binding Rabs are activated by Rab GEFs converting the Rab to its GTP-bound state which allows interaction with any downstream effectors. Finally, the reverse cycle may occur where a GAP is able to return the Rab back to its inactive GDI-bound form where Rab-GDI can extract the Rab from the membrane into the cytosol ready for another cycle (**Figure 1.7**). This cycle is both temporally and spatially controlled, and is one of the key features in Rab membrane regulation (Pfeffer and Aivazian, 2004).

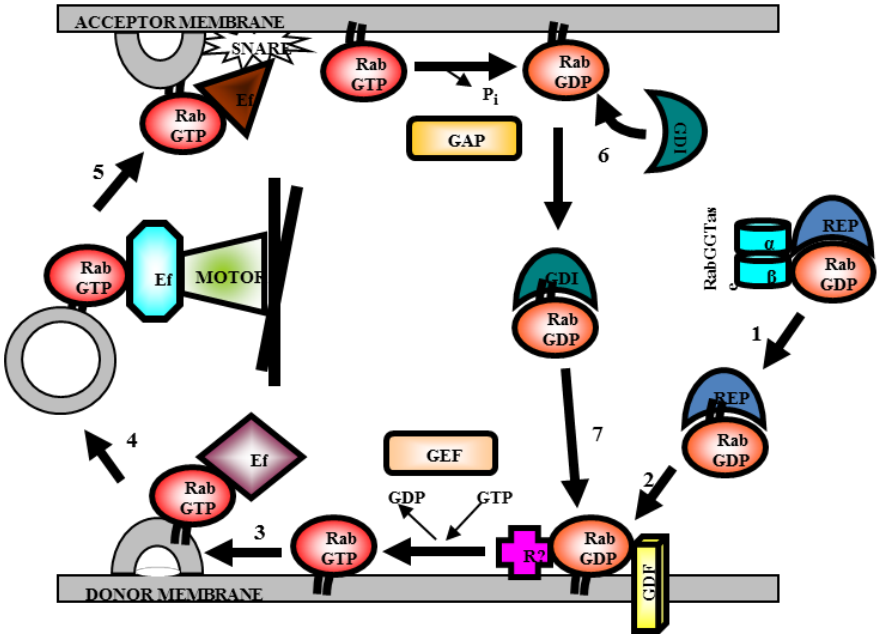


Figure 1.7 – Model of the RabGTPase cycle. After synthesis Rab proteins are recognised by REP which presents the Rab to RabGGTase (Rab geranyl geranyl transferase), the enzyme that prenylates the Rab protein (1). REP then escorts the Rab protein to the appropriate donor membrane where it is recognised by a GDF (GDI-displacement factor) and a putative specific Rab receptor . The GDF destabilises the REP-Rab complex allowing membrane association of the Rab protein (2). The Rab protein is then activated by a GEF (Guanine nucleotide exchange factor) which catalyses the removal of GDP and allows binding of GTP. Once in its active form the Rab protein can interact with multiple effectors (3) to mediate vesicle formation and budding (4), vesicle motility usually through the action of a molecular motor like myosin or kinesin (5) and vesicle docking (5). Rab proteins and their effectors may also interact with SNARE complexes and influence membrane fusion. During or following fusion Rab proteins are inactivated by a GAP (GTPase activating protein) which catalyses the Rab proteins own GTPase activity. Once in its inactive, GDP-bound form the Rab protein can be extracted from the acceptor membrane by GDI (guanine nucleotide dissociation inhibitor) (6). GDI then recycles the Rab protein to its donor membrane where GDF catalyses the dissociation of the Rab-GDI complex and allows membrane association of the Rab protein (7) and the commencement of a new cycle.

Numerous Rab effectors have been identified and characterized (see Table 1 from (Zerial and McBride, 2001)) although many are still unknown. Interestingly, Rab effectors are not structurally conserved proteins implying that these are highly specialized molecules that have adapted to specific organelles and transport systems (Pereira-Leal personal communication).

The mechanism by which Rab proteins achieve their specific intracellular localizations is largely uncharacterized although putative membrane-bound targeting factors, Rab-GEFs, effector binding and lipid composition of membranes have all been implicated. Rabs function by coordinating a continuum of membrane trafficking events in a mechanism termed Rab conversion. This involves controlling the tethering/docking of vesicles to their target membranes, which ultimately leads to their fusion (Markgraf et al., 2007). Rabs also control the vesicle budding and motility, by regulating binding and movement along both the microtubule an actin cytoskeleton. This often involves direct as well as indirect binding with motor proteins such as myosins and dyneins (Pfeffer, 2003)(Pfeffer and Aivazian, 2004).

Due to their functional and membrane specificity, Rab proteins have become membrane identity proteins where each Rab protein is associated with a specialized intracellular compartment (Dacks et al., 2009)(Zerial and McBride, 2001). **Figure 1.8** shows the localization of some of the most well characterizes Rabs and their preferred cellular compartment. Since Rab proteins control vesicle fusion and motility, they have been widely studied in the context of intracellular infections, where many pathogens have evolved the ability to manipulate these proteins in some way in order to invade and survive intracellularly.

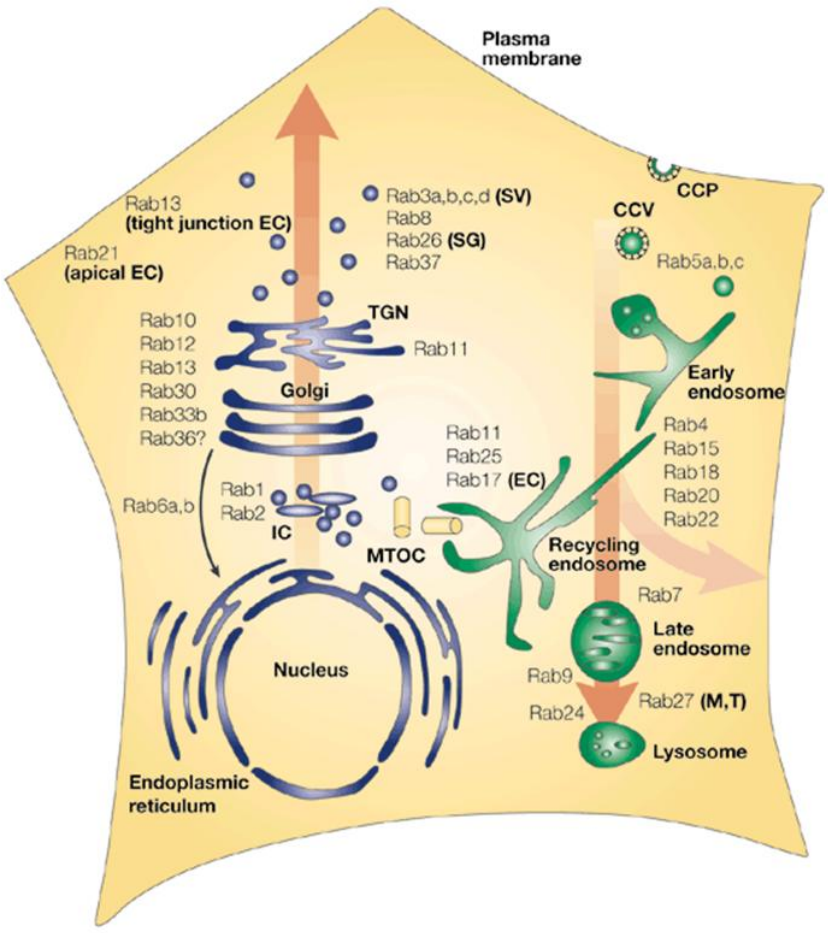


Figure 1.8 – Map of intracellular localization of selected Rab proteins. Summary of the intracellular location of selected Rab proteins in mammalian cells. CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; EC, epithelial cells; IC, ER–Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granules; SV, synaptic vesicles; T, T-cell granules; TGN, *trans*-Golgi nextwork. Image adapted from Zerial & McBride, 2001.

1.7 Phagocytosis and autophagy – the cell´s antimicrobial mechanisms

Mammals have various lines of defense against pathogenic organisms. Most pathogens, in order to establish a successful infection, are able to somehow evade these mechanisms by either passing undetected or by directly

subverting host immune mechanisms to their advantage. At the level of the cell, ingested particles are normally processed by the cell through the endosomal-lysosomal degradation pathway, where pathogens are degraded and digested, and antigens are presented on the surface of the cell to trigger an immune response (Aderem and Underhill, 1999)(Houde et al., 2003). More recently, autophagy has also been described as a pathway used by cells for pathogen degradation and elimination (Levine and Kroemer, 2008)(Subauste, 2009).

1.7.1 Phagosome formation and maturation

Phagocytosis is an essential process that triggers the activation of multiple transmembrane signaling pathways leading to the reorganization of the actin cytoskeleton and the formation of a unique intracellular compartment, termed the phagosome. It is believed that phagocytosis was already present in early eukaryotic ancestors (Dacks and Field, 2007) thus it is not surprising that it is a very tightly regulated endomembrane system.

It is now widely accepted that phagosome maturation occurs through a strictly choreographed sequence of fusion and fission events involving defined compartments of the endocytic pathway. Effective phagocytosis therefore requires two distinct phases; particle internalization and phagosomal maturation. Internalization depends on the type of particle being internalized as well as the target cell, but can be generalized to two general mechanisms; direct, through recognition of specific receptors at the surface of cells by pathogen associated molecules, or indirectly, by opsonins, which trigger specific signaling cascades depending on the nature of the molecules involved. (See (Flannagan et al., 2009) for an excellent review on the subject).

Phagosome maturation, which is the main focus of this project, is one of the first lines of defense against microbial infections. The nascent phagosome quickly matures into an early endosome regulated by Rab5, followed by maturation into a late endosome, a process regulated in part by

Rab7 (Pitt et al., 1992)(Desjardins et al., 1994)(Desjardins, 1995). The late endosome-phagosome then becomes acidified due to the acquisition of the vacuolar proton pump ATPase (vATPase). The phagosome lumen is acidified due to the pumping of hydrogen protons by this protein before fusion with lysosomes, becoming a hydrolase-rich phagolysosome. Here, ingested particles are degraded and processed pathogen antigens are then ready to be presented on the cell surface via recycling endosomes (see **Figure 1.9**).

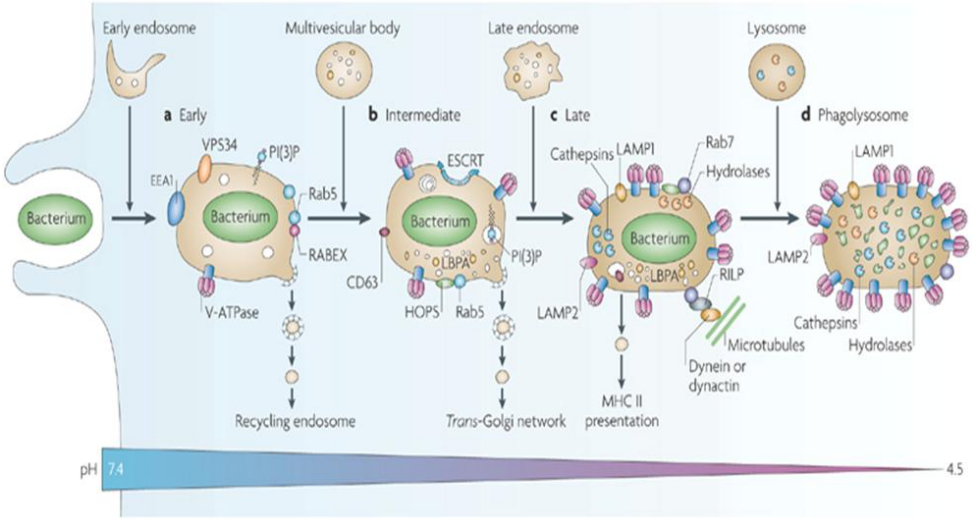


Figure 1.9 – Stages in phagosome maturation. Shortly after pathogen uptake, the phagosome undergoes a series of sequential fusion events with subcompartments of the endocytic pathway. Various stages may be identified, based on a series of protein markers, including an early (a), intermediate (b) and late (c) stage of maturation. The final stage involves fusion with lysosomes, forming a phagolysosome (d) that acquires various hydrolases and becomes acidic, culminating with the degradion of the internalized pathogen. EEA1, early endosome antigen 1; ESCRT, endosomal-sorting complex required for transport; HOPS, homotypic protein sorting; LAMP, lysosomal-associated membrane protein; LBPA, lysobisphosphatidic acid; PI(3)P, phosphatidylinositol-3-phosphate; MHCII, major histocompatibility complex II; RILP, Rab-interacting lysosomal protein. Image adapted from Flannagan et al, 2009.

In professional phagocytic cells such as macrophages, this process may occur within 15 to 30 minutes although this process also occurs in non-professional phagocytes, such as epithelial cells and hepatocytes, but often at a slower rate. Many pathogens have therefore evolved strategies to avoid killing

by phagolysosomes by manipulating these series of sequential fusion events since proper phagosome maturation would ultimately lead to pathogen degradation and elimination (see (Hackstadt, 2000)(Tjelle et al., 2000)(Kahn et al., 2002) for good reviews on the subject).

1.7.2 Autophagy

Macroautophagy (referred to as autophagy hereafter) is an evolutionary conserved catabolic process whereby cytoplasmic materials, including organelles, reach lysosomes for degradation (Mizushima et al., 2008)(Levine and Kroemer, 2008). The metabolic roles of autophagy can be classified in two types: basal autophagy, which occurs at low levels constitutively and is believed to be important in the internal quality control of intracellular components, and induced autophagy, where cells, under nutrient deprivation, self-digest cytoplasmic components to maintain the amino acid pool during stress conditions (Kuma and Mizushima, 2010).

Upon induction, a small vesicular sac, called the isolation membrane or phagophore, is formed and elongates, enclosing a portion of cytoplasm. This result in the formation of a double membrane structure called the autophagosome (see **Figure 1.10**). This vesicle, just like during phagosome maturation, undergoes a series of sequential fusion steps, first with early endosomes and later with late endosomes/ multivesicular bodies (MVBs), forming and intermediate structure called the amphisome, which has all the protein markers of autophagosomes, such as the microtubule-associated protein 1 light chain 3 (LC3) but also markers of the fused organelles, such as Rab7 and LAMP1. These vesicles ultimately fuse with lysosomes, forming autolysosomes, where the internalized components, as well as the inner membrane, are degraded by proteases, lipases, glycosidases and nucleases, all delivered by the lysosome (Mehrpour et al., 2010).

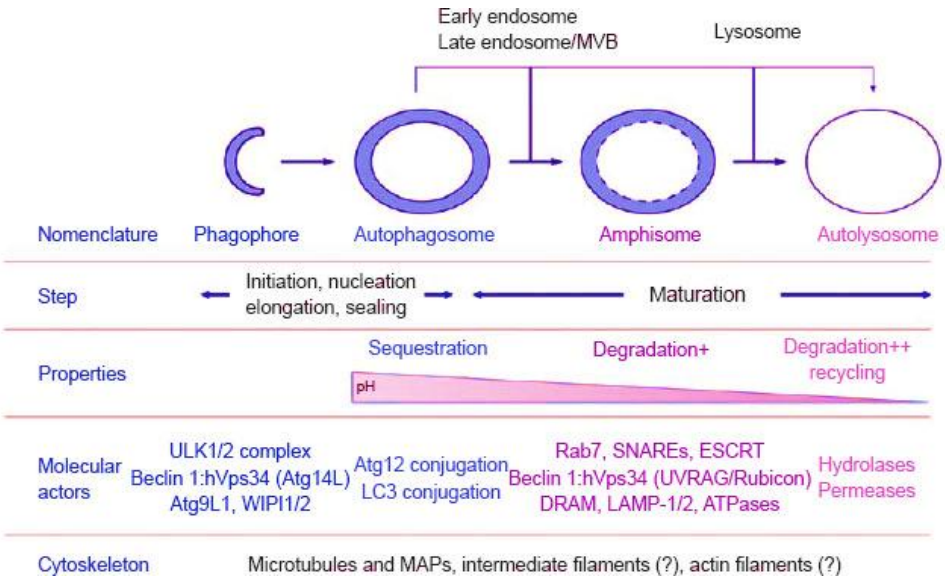


Figure 1.10 – Steps in autophagosome formation and maturation. Autophagy is initiated by the nucleation of a phagophore, which elongates and closes on itself to form and autophagosome. This vesicle then fuses with the endocytic pathway (early and late endosomes and MVBs), in a process termed maturation. The resulting amphisomes is more acidic than the autophagosome and begins to acquire hydrolytic enzymes. This vesicle finally fuses with lysosomes and degradation of the internalized material occurs. Shown in the scheme are some of the molecular players involved at the different stages of this process. Image adapted from Mehrpour et al, 2010.

Various proteins have been described to regulate autophagy. One of the key regulators is TOR kinase, which acts as a major inhibitory signal inhibiting autophagy during normal nutrient conditions (Lum et al., 2005). Downstream of TOR kinase are the family of Autophagy Related Genes (ATGs), first discovered in yeast, which are essential for proper autophagosome formation and maturation (Yorimitsu and Klionsky, 2005). Interestingly, some Rab proteins have also been described to be involved in autophagy. Rab7 has been shown to be required for autophagosomes maturation, stimulating the fusion of autophagosomes with late endosomes/lysosomes (Gutierrez et al., 2004)(Jäger et al., 2004). Interestingly, Rab11 seems to be required for the fusion of autophagosomes and MVBs during starvation conditions (Fader et al., 2008). More recently, Rab1a has also been implicated in the initial formation of autophagosomes (Zoppino et al., 2010)(Huang et al., 2011).

Just like proper phagosome maturation, correct autophagic maturation is an essential pathway for intracellular pathogen elimination, making them two ideal pathways to be manipulated by intracellular pathogens.

1.8 Intracellular pathogens and host cell subversion

Several pathogens have evolved to enter, survive and replicate within mammalian cells. Most of these intracellular pathogens use existing cellular pathways not only to enter host cells, exploiting existing receptors at the surface to attach and enter, but also to acquire nutrients and survive once inside the cell. Because most of these pathways end in the formation of phagolysosomes, which are capable of degrading microorganisms, pathogens have evolved remarkable ways to interact with the host cell phagosome maturation machinery to ensure survival.

Although it seems that each organism seems to have developed its own unique mechanism to evade lysosomal destruction, these strategies may be summarized in five categories: 1) lysis of the phagosomal membrane and escape to the host cytosol, 2) avoidance of the host autophagy pathway, 3) delay of the phagosomal maturation process, 4) subversion of the phagocytic pathway and 5) survival within the harsh phagolysosomal environment (Luzio et al., 2007) (**Figure 1.11** summarizes these alternative strategies used by pathogens to evade host killing). Examples of bacterial and parasite organisms that employ each of these strategies will be discussed as well as the molecular players involved, where known.

It is important to note that many of these pathogens are able to manipulate their host cell in many more ways, such as activating/deactivating apoptotic cell death and/or activating specific protein transcription, although this will not be discussed in this work (Lamkanfi and Dixit, 2010).

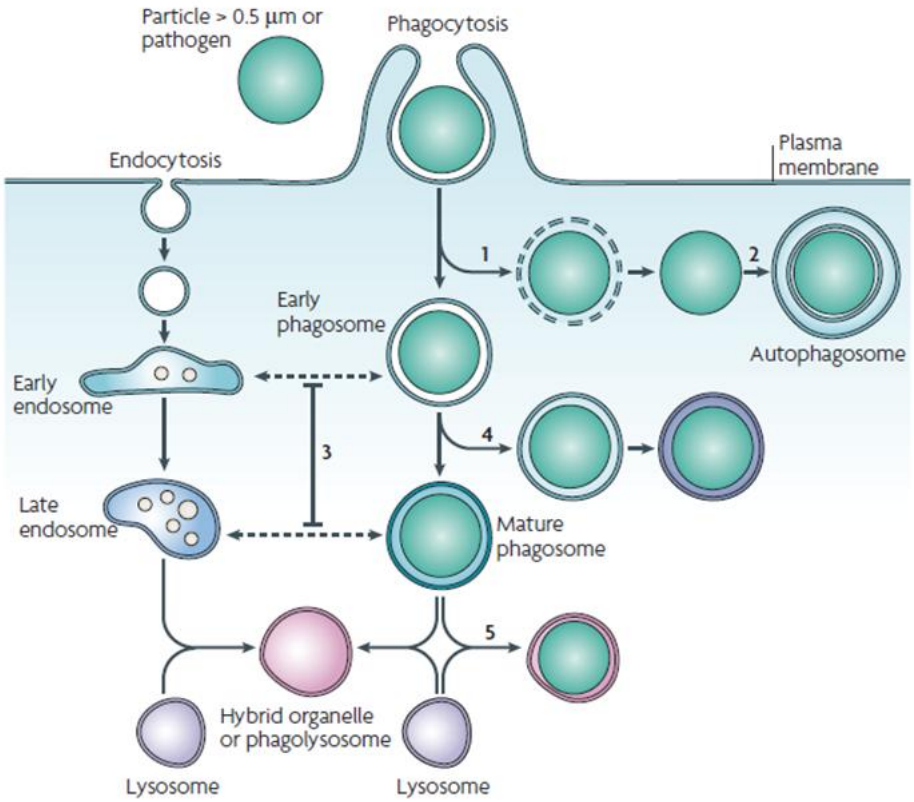


Figure 1.11 – Pathogens survival strategies to avoid lysosomal killing. Pathogens have evolved different strategies to prevent lysosome degradation and killing. These include (1) escape into the cytosol, (2) avoidance of autophagy, (3) delay in phagosomal maturation (4) subversion from the normal phagocytic pathway and (5) ability to survive in the harsh phagolysosomal environment. Image adapted from Luzio et al, 2007.

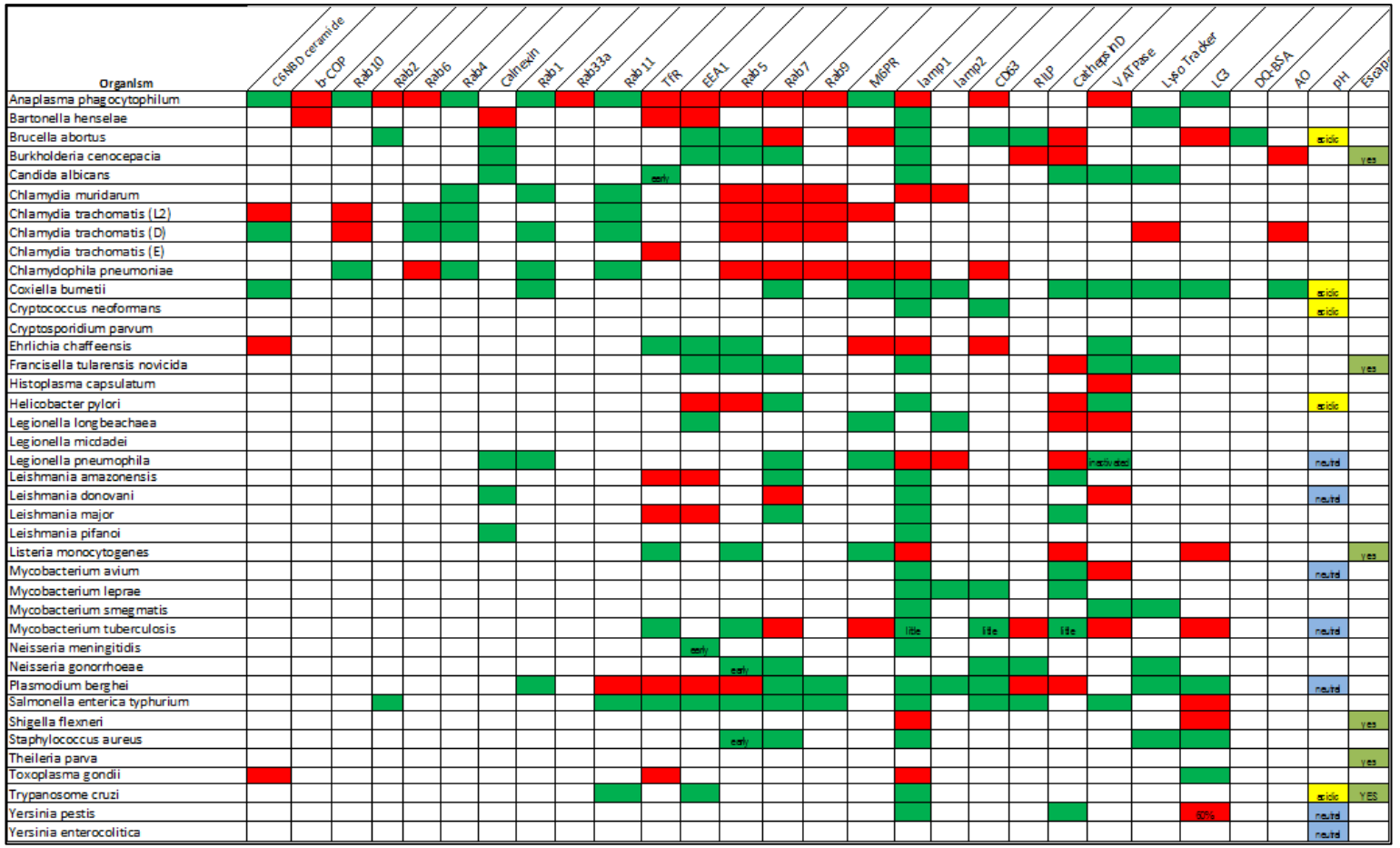
1.8.1 Bacteria manipulation of the host cell

During their intracellular life cycle stage, many bacterial species are able to manipulate their environment in order to survive and disseminate. Many human bacterial pathogens have been extensively studied, and, in some examples, the mechanisms employed have been well characterized (see (Brumell and Scidmore, 2007) for very good reviews on how bacteria pathogens manipulate host Rab proteins).

Figure 1.12 shows a summary table of an extensive list of intracellular bacterial pathogens and specific proteins markers that have been described to be on their respective phagosome membrane. Specific examples will be discussed further below.

In next page:

Figure 1.12 – Intracellular pathogens and membrane markers on their phagosome membrane. Summary table of various intracellular bacterial and parasites pathogens and various protein markers described to be on their phagosome membrane. Red boxes represent markers NOT found on the membrane, green boxes represent marker found on the membrane and white boxes represent missing data. Yellow boxes represent acidic phagosomes while blue boxes represent neutral phagosomes. Pale green boxes in the last column show pathogens that, upon invasion with the formation of a vacuole membrane around them, later escape and survive in the host cytosol. The results obtained in the context of this project, related to membrane markers found surrounding *Plasmodium berghei* PVM are also included. References from this image are listed in **Supplementary Figure 1**.


Organism
C6NBD ceramide
br COP
Rab10
Rab2
Rab6
Rab4
Calnexin
Rab1
Rab33a
Rab11
TfR
EEA1
Rab5
Rab7
Rab9
M6PR
lamp1
lamp2
CD63
RILP
Cathepsin D
V ATPase
Lyso Tracker
LC3
DQ-BSA
AO
pH
Escape
Anaplasma phagocytophilum
Bartonella henselae
Brucella abortus
Burkholderia cenocepacia
Candida albicans
Chlamydia muridarum
Chlamydia trachomatis (L2)
Chlamydia trachomatis (D)
Chlamydia trachomatis (E)
Chlamydophila pneumoniae
Coxiella burnetii
Cryptococcus neoformans
Cryptosporidium parvum
Ehrlichia chaffeensis
Francisella tularensis novicida
Histoplasma capsulatum
Helicobacter pylori
Legionella longbeachaea
Legionella micdadei
Legionella pneumophila
Leishmania amazonensis
Leishmania donovani
Leishmania major
Leishmania pifanoi
Listeria monocytogenes
Mycobacterium avium
Mycobacterium leprae
Mycobacterium smegmatis
Mycobacterium tuberculosis
Neisseria meningitidis
Neisseria gonorrhoeae
Plasmodium berghei
Salmonella enterica typhurium
Shigella flexneri
Staphylococcus aureus
Theileria parva
Toxoplasma gondii
Trypanosome cruzi
Yersinia pestis
Yersinia enterocolitica
early
late
inactivated
50%
acidic
neutral
yes
YES

As mentioned previously, one mechanism used by bacteria to survive in host cells is to enter the host cell with the formation of a vacuole around it, but quickly escape to the host cytosol. This is the case with *Listeria, Burkholderi, Francisella, Shigella* and *Rickettsia* species. *Listeria monocytogenes* initially inhabits a membrane bound vacuole that acquires markers of early endosomes such as Rab5, but later secretes a pore-forming toxin called Listeriolysin O which causes a delay in phagosomal maturation by causing alterations in both vacuolar pH and Ca^{2+} concentration, giving the bacteria time to escape into the host cytosol (Henry et al., 2006). Once in the cytosol, *Listeria* is able to escape autophagic death by secreting ActA protein, which recruits the Arp2/3 complex and Ena/VASP to the bacteria surface, disguising it from autophagic recognition (Yoshikawa et al., 2009). Remarkably, *Ricketssia conorii* also secretes an ActA related protein, RickA, a protein that recruits the host Arp2/3 complex, inducing actin nucleation and generation of actin filaments, but possibly, also protecting it from autophagic killing (Gouin et al., 2004)(Jeng et al., 2004).

Shigella flexneri is also able to evade autophagic delivery to the lysosome after escaping to the cytosol by a secreting IcsB, which avoids bacterium autophagic death, although the exact mechanism by which IcsB achieves this is still largely unknown (Ogawa et al., 2005)(Kayath et al., 2010). Curiously, *Burkholderi pseudomallei*, another intracellular bacteria, secretes BopA, which is a IcsB homologue, which also contains the essential cholesterol binding domain (CBD) required for autophagic escape (Kayath et al., 2010).

Another common mechanism used by bacteria is to delay phagolysosome biogenesis, a process that has been extensively studied in *Salmonella* and *Mycobacteria* infections. In the case of *Salmonella enterica*, phagosome maturation is inhibited due to the manipulation of specific proteins recruited to the vacuole membrane, such as Rab proteins. *Salmonella* resides in an acidified late-endosome-like compartment, positive for Rab7, Rab9 and

LAMP1 (Steele-Mortimer et al., 1999), but is able to uncouple Rab7 from its effector RILP (Rab Interacting Lysosomal protein) by the secreted protein SifA, preventing its centripetal displacement and fusion with lysosomes (Harrison et al., 2004)(Jackson et al., 2008).

Mycobacteria tuberculosis acquires Rab5 but inhibits Rab7 acquisition on its phagosome, inhabiting an early-endosome-like compartment and therefore avoiding Rab7-RILP mediated fusion with lysosomes (Sun et al., 2007). The *M.tuberculosis* phagosome acquires LAMP2 and CD63 markers but remains in a mildly acidic compartment, probably as a consequence of inhibiting vATPase recruitment to the phagosome membrane (Sturgill-Koszycki et al., 1994) and also any markers for mature lysosomal hydrolases, such as Cathepsin D are absent (Ullrich et al., 1999). *M.tuberculosis* also produces lipids that mimic mammalian phosphatidylinositols and inhibit phosphatidylinositol 3- phosphate (PI3P)-dependent trafficking pathways, including the acquisition of the Rab5 effector Early Endosome Antigen 1 (EEA1) causing a phagosome maturation block (Fratti et al., 2001)(Fratti et al., 2003)(Vergne et al., 2004).

Legionella pneumophilia is an example of a pathogen that is able to avoid fusion with the endocytic pathway during the first hours of infection, inhibiting vacuolar ATPase activity by secreting a protein, SidK, that specifically targets host vATPase (Xu et al., 2010). At the same time, *L.pneumophilia* intercepts the host ER-to-Golgi vesicle traffic (Tilney et al., 2001)(2001 Tilney) and its phagosome retains ER markers. This occurs by the secretion of SidM/DrrA protein, which acts as a guanine nucleotide dissociation inhibitor displacement factor for Rab1 (Murata et al., 2006). At the same time, another region of SidM/DrrA activates Rab1 by functioning as a guanine exchange factor (GEF). In this way, this bacterial protein competes with endogenous GEFs to recruit and activate Rab1 on the bacterial vacuole membrane.

Another *Legionella pneumophila* protein, LepB, was found to inactivate Rab1 by stimulating GTP hydrolysis, regulating removal from the

membrane (Ingmundson et al., 2007). These proteins, along with others such as SidJ which is involved in recruitment of ER proteins to the bacteria vacuole, are all involved in transforming the *Legionella* vacuole into an ER-derived phagosome which allows for pathogen growth and development (Liu and Luo, 2007).

Finally, some pathogens, instead of trying to avoid the harsh phagolysosomal compartment, actually require this unique environment to survive inside host cells. This is the case with *Coxiella burnetii*, a pathogen that has evolved not only to survive but to thrive within a phagolysosome. Following internalization, *Coxiella*-containing phagosomes sequentially recruit Rab5a and Rab7a (Heinzen et al., 1996) and later appear positive for Cathepsin D and LC3, the autophagic marker (Berón et al., 2002)(Romano et al., 2007).

1.8.2 Apicomplexa pathogen manipulation of the host cell

Just like bacteria, intracellular protozoan pathogens also show a superb array of strategies to interact with the host cell. While escaping immune surveillance and accessing intracellular nutrients, protozoa have evolved various mechanisms to subvert host cell processes and pathways (Plattner and Soldati-Favre, 2008). Included in **Figure 1.12** are intracellular Apicomplexa parasites where the composition of the phagosomal membrane is known. Note that, although most of the organisms included are either medically or economically important, for most of them, much data is lacking, demonstrating that more resources should be employed to understand these pathogens better in order to find more efficient elimination strategies.

Pathogen survival depends on the ability of parasites to establish their intracellular niche, and just like bacteria, parasites either survive inside a membrane bound compartment or in direct contact with the cytosol. Only a few parasites employ this second strategy, where, after entering into the host cell within a membrane bound vacuole they quickly escape to the cytosol by lysing

this membrane. *Trypanosoma cruzi* and *Theileria* species utilize this mechanism to escape destruction by phagolysosomal degradation. The exit of *T.cruzi* from the vacuole involves the secretion of Tc-TOX protein, which has pore forming activity at acidic pHs (Andrews et al., 1990)(Ley et al., 1990).

Theileria annulata on the other hand, does not require an acidic vacuole to escape to the cytoplasm, however escape to the cytoplasm coincides with the discharge of the parasite apical organelles, namely the rhoptries and microspheres, although their exact protein content remain unknown (Shaw, 2003).

Leishmania donovani is an interesting case of a parasite that applies different intracellular strategies depending on the stage of its life cycle. *L.donovani* promastigotes inhibit phagosome-endosome fusion by a unique lipophososphoglycan (LPG) on their surface (Miao et al., 1995)(Descoteaux and Turco, 1999). On the other hand, after amastigote transformation, lysosome fusion is not inhibited and the vacuole acidifies by fusion with endosomes and lysosomes, an event required to trigger replication (Antoine et al., 1990)(Joshi et al., 1993)(Antoine et al., 1998).

Toxoplasma gondii resides in a vacuole that is not acidified and resists lysosome fusion (Jones and Hirsch, 1972)(Sibley et al., 1985). Initial evidence from electron microscopy revealed that both antibody-coated and dead parasites readily fused with electron-dense labeled lysogenic vacuoles, while those containing live parasites where resistant to lysosomal fusion (Jones and Hirsch, 1972)(Jones et al., 1972).

T.gondii vacuole membrane is formed rapidly *de novo* at the time of parasite entry and derives from both the host plasma membrane and the secretion of rhoptry-derived secretory vesicles (Håkansson et al., 2001). It was initially proposed that parasite proteins secreted to the PVM were responsible for the blockage of vacuole acidification and lysosome fusion (Sibley et al., 1985)(Sibley et al., 1986). However, it was later shown that once the PVM was formed, resistance to fusion and acidification was not dependent on live parasite, since vacuoles containing parasites killed post invasion were still

devoid of any signs of lysosomal fusion (Nguyen et al., 1978)(Joiner et al., 1990). This suggested that the PVM is inherently resistant to lysosome fusion, and this is established at the time of parasite invasion.

While resisting fusion with lysosomes, *T.gondii* vacuoles also associate with host microtubules (Melo et al., 2001), intermediate filaments (Halonen and Weidner, 1994) and endoplasmic reticulum (Jones and Hirsch, 1972). Subsequent work showed that *T.gondii* ROP2 protein, which is secreted from the rhoprty during parasite invasion and associates with the PVM, was involved in PVM-host organelle associations (Beckers et al., 1994)(Sinai and Joiner, 2001)(See (Boyle and Radke, 2009) for a good review).

Just like with bacterial pathogens, the vacuole membrane is the interface between host and parasite, playing a role in nutrient acquisition by the parasite from the host cell. It seems clear that intracellular parasites have evolved various types of intracellular life styles. The questions still remains how these eukaryotic organisms are able to acquire nutrients, especially when they have evolved non-fusogenic vacuole membranes. There are two types of mechanisms known to be used by parasites for nutrient acquisition; either making their PVM permeable to certain molecules or by developing specialized "feeding organelles".

Toxoplasma and *Eimeria* are both examples of parasites with PVMs that are freely permeable to small cytosolic molecules through the introduction of high capacity channels (Schwab et al., 1994)(Werner-Meier and Entzeroth, 1997). This suggests that apart from acting as a barrier to fusion with host vesicles, the PVM also functions as a molecular sieve, allowing for the transfer of nutrients from the host cytoplasm, essential for parasite growth. The same occurs with *Plasmodium* erythrocytic stages (Desai et al., 1993)(Desai and Rosenberg, 1997) while a more recent study by Bano et all. (2007) has shown that the PVM of *Plasmodium* liver stages also contains non-selective channels which allow the passage of molecules up to 850Da, suggesting that a similar passive "feeding" mechanism may have evolved in these species. Interestingly, the same group identify a new "feeder organelle" termed the Host Organelle-

Sequestering Tubulo-structures (or H.O.S.T.) which deliver endo-lysosomes in a unidirectional transport mechanism in *Toxoplasma gondii* (Coppens et al., 2006). This structure is organized by deep microtubule based invaginations of the PVM which serve as conduits for the transport of host vesicles into the vacuolar space, providing a new nutrient acquisition mechanism. Further studies would be required to establish if this newly discovered "organelle" is present in other species.

Leishmania on the other hand, does not contain such pores or channels on its vacuole membrane (Antoine et al., 1990) but instead seems to import nutrients via fusion of endosomes and lysosomes with its PVM during the amastigote stage, providing a relatively constant supply of nutrients.

Since very little is known about *Plasmodium* liver stage host interactions and the possible source of nutrients for parasite growth and development, part of this project involved looking at this in more detail.

1.9 Aims and objectives

The liver stage is still the most elusive stage of the entire life cycle of the *Plasmodium* parasites. Although it was identified more than 50 years ago, technical constrains have made it very difficult to study the molecular mechanisms involved in this stage

The dogma in the field is that *Plasmodium* parasites in the liver are able to avoid interactions with the host hepatocyte during this stage of infection (Bano et al., 2007). Early experiments in our laboratory suggested otherwise. Preliminary studies where LAMP1 staining was performed in infected cells, showed a very clear aggregation of late endosomes/lysosomes around the parasite. This led us to propose that host-parasite interactions were occurring. The fact that a liver stage vaccine is possible (Nussenzweig et al., 1967) also suggests the existence of a strong interaction between the parasite and the host liver cells during this stage of *Plasmodium* infection, since the immune system is able to detect parasites and eliminate them in pre-immunized mice. Thus we proceeded to analyze any possible interactions between the Parasitophorous Vacuole membrane and host organelles in more detail.

Interestingly, in one of the first articles characterizing *P.berghei* liver stage, using electron microscopy, the authors note that "primary lysosomes are clearly visible at the parasite-host interface" and that "vacuoles of different sizes but containing similar material were also arranged at the periphery of the parasite; evidence was found to indicate that their contents are secreted into the PV (Meis et al., 1983). This intriguing observation led us to speculate that some uknown interaction between *Plasmodium* parasites and the host endo-lysosome systems could be occurring, which we proceeded to investigate in the course of this project.

The overall goal of this research project was to identify any host-parasite interactions during *Plasmodium berghei* infection within hepatocytes. This goal was divided into various stages:

(i) Study the interaction between host organelles and the parasite, using mainly immunofluorescence techniques, the results of which are shown in Chapter 2

(ii) Study the role of host trafficking molecules during *Plasmodium* invasion using a siRNA screen approach, which is discussed in Chapter 3.

(iii) Dissect the role of lysosomes during *Plasmodium* liver infection, discussed in Chapter 4.

Studying the liver stage of *Plasmodium* development, and specially the host-parasite interactions involved in this stage, could uncover interesting and unique therapeutic targets for parasite disruption. Interestingly, the passage from the mosquito´s salivary glands to the mammalian liver is a very striking bottleneck in the *Plasmodium* lifecycle and thus presents a perfect target for disease interventions.

2. Characterization of *Plasmodium* liver development and host interactions

All the work presented in this chapter was performed by Mafalda Lopes da Silva with the help of Carolina Matos with the exception of the Electron Microscopy sample preparation, which was done in collaboration with Silene Wavre, Imperial College.

Summary

Plasmodium sporozoites, once inside the liver, must invade and remain within a hepatocyte cell long enough for the first replicative stage within the mammalian host to be accomplished. Upon migration through various cells, sporozoites are able to actively enter hepatocytes, forming a Parasitophorous Vacuole Membrane (PVM) around it. Once this intracellular niche is established, parasite replication and growth is initiated. Dramatic morphological as well as gene expression modification occur at this stage, and the parasite is able to achieve one of the highest replication rates known within eukaryotic species (Sinnis and Sim, 1997). The kinetics of parasite development in hepatocytes was defined while analyzing the morphological changes associated with each stage of parasite development. An analysis of parasite size increase throughout liver development was performed in order to establish the tools required for the remainder of the work presented in this thesis.

Once inside the hepatocyte, *Plasmodium* parasites encounter a multitude of host organelles, vesicles and proteins. Host proteins known to be involved in the endomembrane system were analyzed for possible interactions with the parasite at various stages of *Plasmodium* liver infection, using indirect immunofluorescence. *Plasmodium* parasites were seen closely associated with host endoplasmic reticulum and the Golgi apparatus. No interaction with host peroxisomes, early and recycling endosomes was observed, but surprisingly, host late endosomes and lysosomes were seen to significantly aggregate around the parasite, throughout its entire liver stage development. Disrupting late endosome/lysosome function by depleting proteins known to be involved in late endosome/lysosome structure and function using specific siRNA did not affect parasite invasion or development, suggesting a more complex host-parasite interaction which will be characterized further in subsequent chapters.

Materials & Methods

Cell lines and culture conditions

Mouse hepatoma cell line Hepa1-6 was generously provided by M.M.Mota (IMM, Lisbon). HEK293A cells lines were kindly provided by J.Ramalho (FCM, Lisbon). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen) supplemented with 10% Fetal Calf serum (FCS) (Gibco/Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/Invitrogen). Cells were maintained in a humidified cabinet at 37 ^{0}C and 10% CO_2.

Primary mouse hepatocyte isolation and culture

Primary mouse hepatocytes were isolated from Female C57BL/6 mice (8-10weeks) according to the protocol described in (Gonçalves et al., 2007). Hepatocytes were isolated by perfusion of mouse liver lobules with liver perfusion medium and liver digest medium (Gibco/Invitrogen) followed by purification using a 1.12 g/ml, 1.08 g/ml and 1.06 g/ml Percoll gradient. Cells were counted and plated ($1x10^5$) on coverslips coated with 0.2% Gelatin in PBS. Cells were cultured in William´s E medium with 4% FCS, 1% penicillin-streptomycin, 50 mg/ml of Epidermal Growth Factor (EGF), 10 μg/ml transferrin, 1μg/ml insulin, 3.5 μM hydrocortisone and maintained at 37^{0}C and 5% CO_2.

All mice were bred and housed at the Instituto Gulbenkian de Ciência animal house facility and Animal handling was conducted according to institutional animal care and use committee-approved protocols.

Parasite strains and cultivation

P. berghei ANKA and Green fluorescent protein (GFP) expressing *P. berghei* ANKA (parasite line 259cl2) (Franke-Fayard et al., 2004) salivary gland sporozoites were collected from infected female in *Anopheles stephensi* infected mosquitoes. *Anopheles stephensi* mosquitoes were bred in the insectariums of the Instituto de Medicina Molecular, Lisbon. Mosquitoes infected with *P. berghei or* GFP-*P.berghei* parasites were dissected between days 20 and 24 days after the infectious blood meal. Mean number of sporozoites were determined using a hemocytometer. Parasites were added to wells and plates were centrifuged at 3000rpm for 5 minutes at 4ºC for parasites to come into contact with the cells.

Microscopy sample preparation

Hepa1-6 cells ($9x10^4$) were seeded on coverslips and once sample were ready to be fixed, cells were washed 2x with PBS, and fixed with either 4% Paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS for 15 minutes at room temperature. Cells were washed 2x in PBS and incubated for 10 minutes with 10nM NH_4Cl to quench the PFA. Cells were washed 2x in PBS and permeabilized with 1% BSA (Bovine Serum Albumin, Sigma), 0.05% saponin (Sigma) and 1% FCS (Gibco/Invitrogen) in PBS. Cells were incubated with primary antibodies in the same solution and incubated for 1 hour. After washing in PBS, cells were incubated with secondary antibodies in the same solution for 30 minutes at room temperature. To visualize the nucleus, cells were incubated with DRAQ5 (1:300 in PBS) (Biostatus Limited) for 10 minutes or DAPI (Invitrogen) for 1 minute. Samples were mounted using MOWIOL mounting medium (Calbiochem).

Antibodies used

Antibody	Source	Catalogue number	Raised in	Fixed with	Dilution used
α-2E6	M.M.Mota		mouse	PFA	1:2500
α-UIS4	M.M.Mota		rabbit	PFA	1:500
α-EEA1	Sigma	E3906	rabbit	PFA	1:500
α-TfR	Zymed	13-6800	mouse	PFA	1:100
α-CD63	BD Pharmingen	551458	mouse	PFA	1:100
α-LAMP1	Hybridoma Bank*	1D4B	rat	PFA	1:300
α-PMP70	Sigma	P0497	rabbit	PFA	1:1000
α-Calnexin	Sigma	C4731	rabbit	PFA	1:500

Table 1 - Antibodies used in Chapter 2. *Developmental Studies Hybridoma Bank, University of Iowa.

Live cell imaging

Hepa1-6 cells ($4x10^4$) were seeded on glass bottom culture dishes (MatTek Corporation) and infected with $5x10^4$ GFP-*P.berghei* sporozoites. Samples were visualized using an inverted Leica SP5 confocal microscope with a resonance scanner and fitted with a temperature and CO_2 control chamber.

Virus production and cell transduction

cDNA of mouse Rab proteins, including the UTR, were amplified from total RNA extracted from cells (see Supplementary Table 2) using RNeasy Mini kit (Qiagen) or from existing EST clones as shown in Supplementary Table 2. Reverse transcription reaction was done using SuperScriptII (Invitrogen) using Poly dT primers according to manufacturer´s protocols (Invitrogen). Amplification by PCR, using Phusion polymerase (New England Biolabs, Hitchin, UK), using specific primers with restriction sites included in

the design (Supplementary Table 2) and according to manufacturer´s protocols. Fragments were cloned into the mammalian expression vector pEGFP vector (Lopes and Ramalho 2007) using the restriction sites included in the primers. LR reaction to insert into viral vector pAd/BLOCK-iT-DEST Gateway vector was performed according to manufacturer's protocols (Invitrogen). Recombined DNA was isolated using QIAGEN Plasmid Midi kit (Qiagen) and 5µg of DNA was digested with PacI (New England Biolabs, UK) according to manufacturer's protocols, followed by purification by phenol/chloroform and precipitated using isopropanol. HEK293A cells were grown to 90% confluency in 25cm^2 flasks and were transfected with 1-2mg of DNA using Lipofectamine 2000 Reagent (Invitrogen). Cells were left until virus production was observed by monitoring GFP fluorescence, plaque formation and cell death. The supernatant was collected and three freeze-thaw cycles were performed. Viruses were re-amplified by infection new HEK293A cells in a 25cm^2 flask using 100µl of supernatant and monitoring GFP fluorescence in the cells. Three freeze-thaw cycles of the supernatant from the re-amplified virus was performed and aliquots were maintained at -80ºC for storage.

Viral titres required to give GFP expression in 90% of Hepa1-6 cells were calculated and adequate virus amounts were added to Hepa1-6 cells the day before infection with parasites, or as indicated in particular experiments. Suitable gene expression was usually obtained after 16-24 hours of virus addition.

Virus constructs used in this chapter: GFP-Rab5a, GFP-Rab7a, GFP-OSBP (see **Supplementary Table 2** for details).

EM sample preparation

8x10^4 Hepa1-6 cells on coverslips were infected (1x10^5 sporozoites) and fixed 24 hours post infection in 2% paraformaldehyde and 2% gluteraldehyde in 0.1M cacodylate buffer for 30 minutes. After washing in 0.1M cacodylate buffer, cells were postfixed in 1.5% potassium ferricyanide

and 1% Osmium tetroxide for 1 hour on ice. The cells were subsequently incubated in 1% tannic acid in 0.05M Sodium cacodylate for 45 minutes and dehydrated in ethanol (70%, 90% and absolute). Coverslip were then transferred to 1:1 propylene oxide: Epon for 1 hour, followed by two changes and embedding in Epon.

Ultra-thin sections were stained with lead citrate before examination on a JEOL 1010 transmission electron microscope (Welwyn Garden City, United Kingdom). Images were taken with a Gatan OriusSC100B charge-coupled device camera and analysed with Gatan Digital Micrograph and Adobe Photoshop.

siRNA transfection

For 24-well plates, 100nM of gene specific siRNA pools (Dharmacon) were added to 100µl of Optimem (Gibco/Invitrogen). 1.2µl of Oligofectamine (Invitrogen) was added 100µl to Optimem. After 5 minutes of incubation at room temperature, these two mixtures were combined, mixed gently, and incubated for 20 minutes at room temperature. Growth medium was removed from cells ($5x10^4$) seeded the day before transfection, and the siRNA mixture was added. Cells were incubated for 3 hours at 37ºC and then the medium was changed to growth medium. Cells were infected with parasites ($5x10^4$) 48 hours post siRNA addition. See Supplementary Table 1 for siRNA sequences.

Gene specific expression quantification by qRT-PCR

Total RNA was isolated from cells using an RNeasy Mini kit (Quiagen) and was converted into cDNA using SuperScript® II (Invitrogen) according to manufactures protocols, using random primers. qRT-PCR reactions were performed using an ABI Prism 7900HT system (Applied Biosystems) using SybrGreen reagent. 5µl of SybrGreen, 4µl of cDNA sample together with 1µl of adequate primers was used per well, in triplicate

conditions. For each protein, gene expression was calculated relative to control wells and standardized using α-Tubulin as a housekeeping gene.

The following primers were used in this Chapter for qRT-PCR: Mouse Rab7a primers (sense, 5′- ccccaacactttcaaaaccc-3′, and antisense, 5′-tggcccggtcattcttgtcc-3′), mouse α-Tubulin primers (sense, 5′–ggtggatctagaacct-3′ and antisense, 5′– cccagtgagtgggtcagc-3′).

Infection rate and EEF size quantification

Infection rate, unless otherwise stated, is the total number of parasite in one well/coverslip. Nuclei were also counted to ensure similar cell confluency in all samples.

To measure exo-erythrocytic form (EEF) size, between 20 and 30 images of EEFs for each condition were taken using an SP5 confocal microscope. The circumference of each EEF was draw manually, and EEF area (in μm^2) was calculated automatically using ImageJ (NIH).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software Inc.) using an unpaired Student t test. $P<0.05$ were considered statistically significant.

2.1 Morphological modification and growth kinetics during *Plasmodium berghei* liver development

Before *Plasmodium berghei* sporozoites invade a liver cell and establish infection, they need to penetrate and migrate through various cells in order to be activated for infection (Mota et al., 2001). This leads to non-synchronous infection as some sporozoites become competent for infection before others. Thus, for each time post infection, a heterogeneous population of parasite forms and sizes may be found. This is shown in **Figure 2.1** and **Figure 2.2**, where sporozoites were added to Hepa1-6 cells and cells were fixed at various times post infection. Parasites were stained with 2E6 (*Plasmodium* anti-HSP70 protein) (red) and anti-UIS4 antibodies, staining the parasite cytoplasm and the Parasitophorous Vacuole Membrane (PVM) (green), respectively.

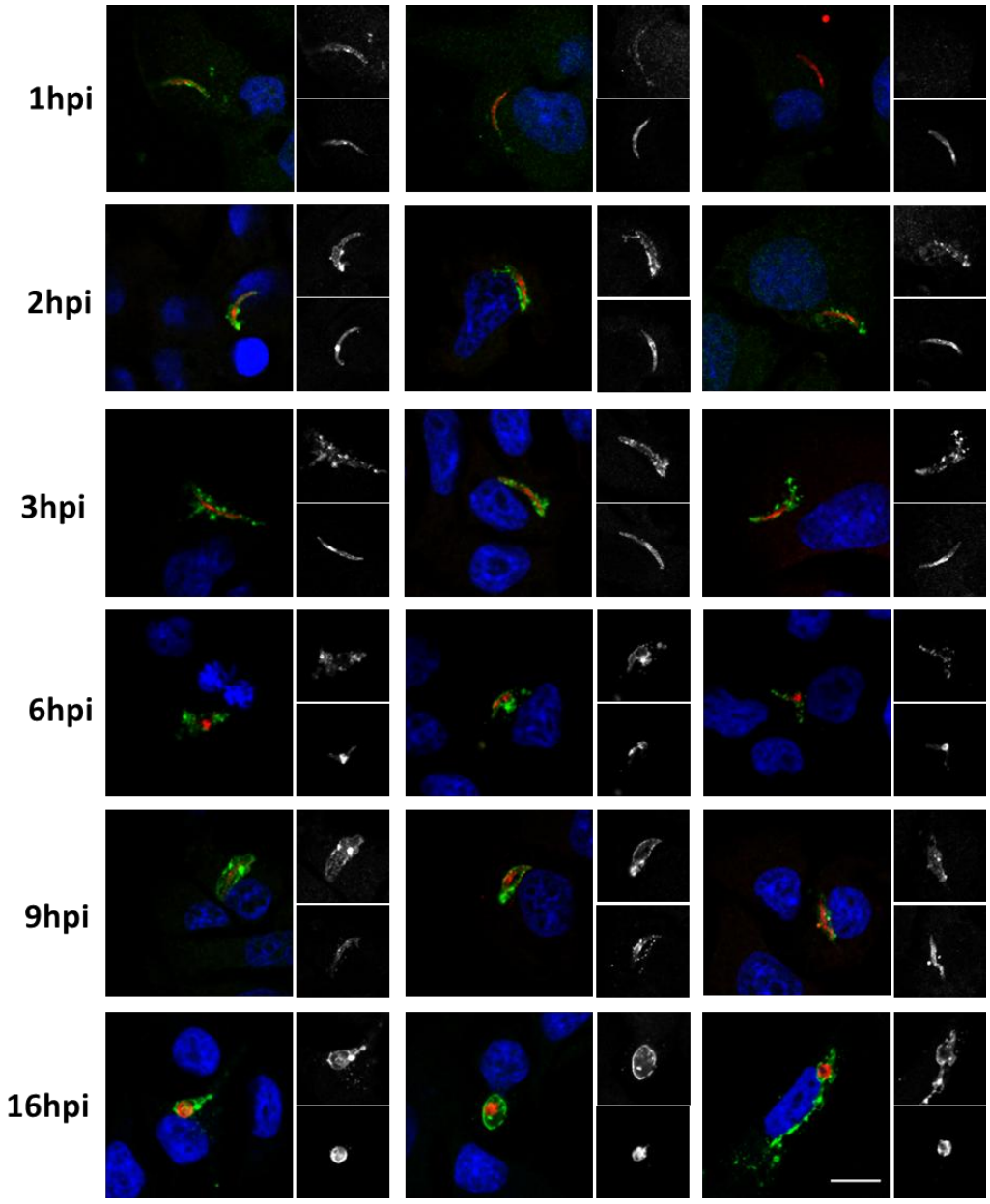


Figure 2.1 - Initial development of Plasmodium berghei parasites in Hepa1-6 cells. Hepa1-6 cells were infected with *P.berghei* parasites and infection was stopped at different hours post infection (hpi). Three representative images for each time of infection are shown. Samples were where stained with 2E6 antibody to stain for the parasite cytoplasm (red and lower right panels) and anti-UIS4 antibody, a parasite protein targeted to the PVM (green and upper right panels). Nuclei were stained with DRAQ5 (blue). Scale bar:10µm.

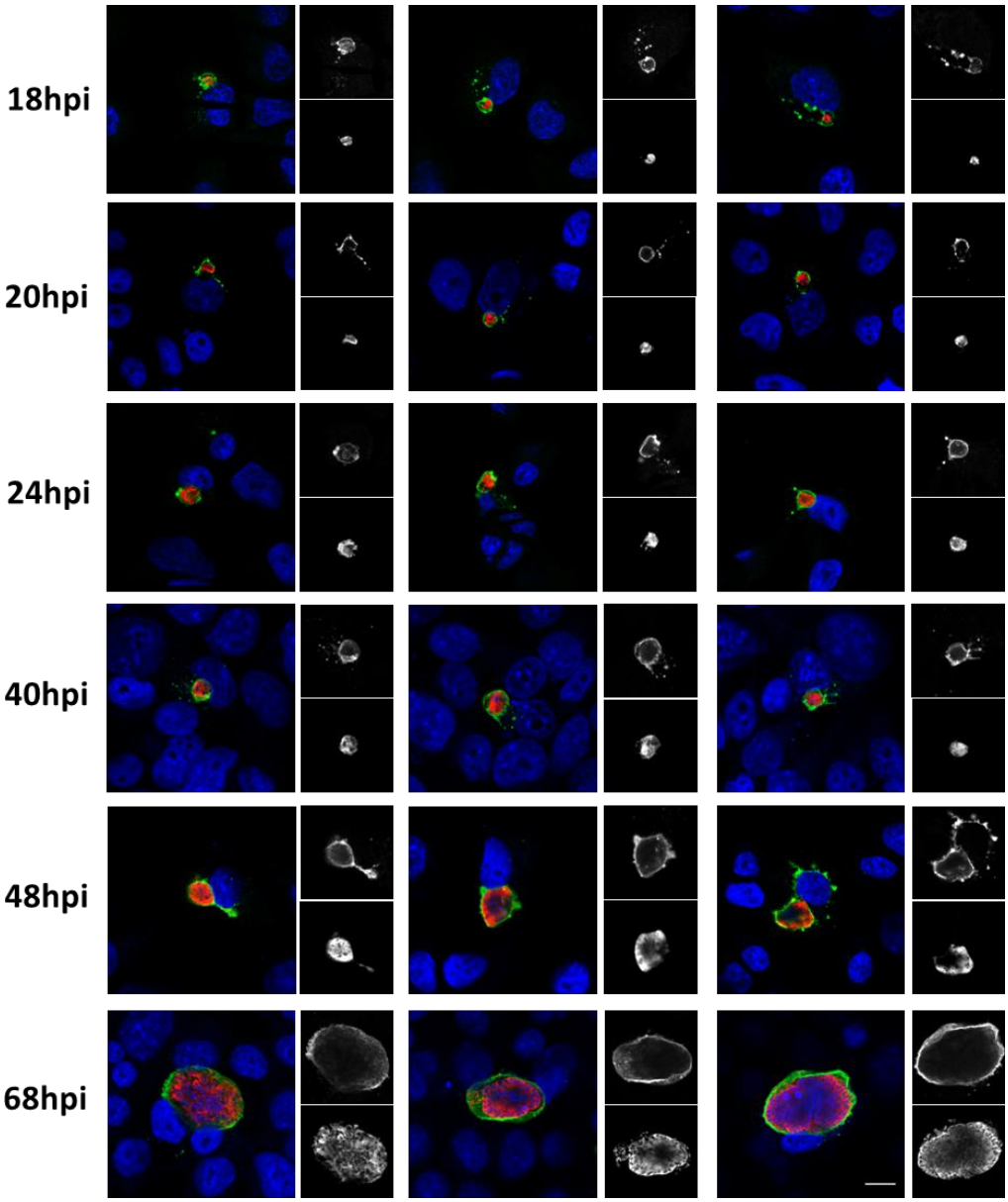


Figure 2.2 - Late development of Plasmodium berghei parasites in Hepa1-6 cells. Hepa1-6 cells were infected with *P.berghei* parasites and infection was stopped at different hours post infection (hpi). Three representative images for each time of infection are shown. Samples were where stained with 2E6 antibody to stain for the parasite cytoplasm (red and lower right panels) and anti-UIS4 antibody, a parasite protein targeted to the PVM (green and upper right panels). Nuclei were stained with DRAQ5 (blue). Scale bar:10μm.

Three representative images are shown for each time point, where it is clear to see that there is some heterogeneity in the parasite form at each stage of liver infection. This heterogeneity is maintained throughout the entire liver stage development, as seen from the images in **Figure 2.2**. Similar growth kinetics was observed in isolated mouse primary hepatocytes (See **Figure 2.11**).

Plasmodium sporozoites undergo dramatic morphological changes in a very short period of time. During the first hour of infection, parasites maintain their characteristic elongated form, but quickly start to form a round bulb in the center, which quickly increases in size. The remaining elongated terminal ends are often still seen up to 9 hour post infection, but ultimately disappear. The initial passage from the elongated to the spherical form was also observed using live cell imaging using GFP-*P.berghei* parasites where GFP is constitutively expressed in the cytoplasm and is shown in **Figure 2.3**.

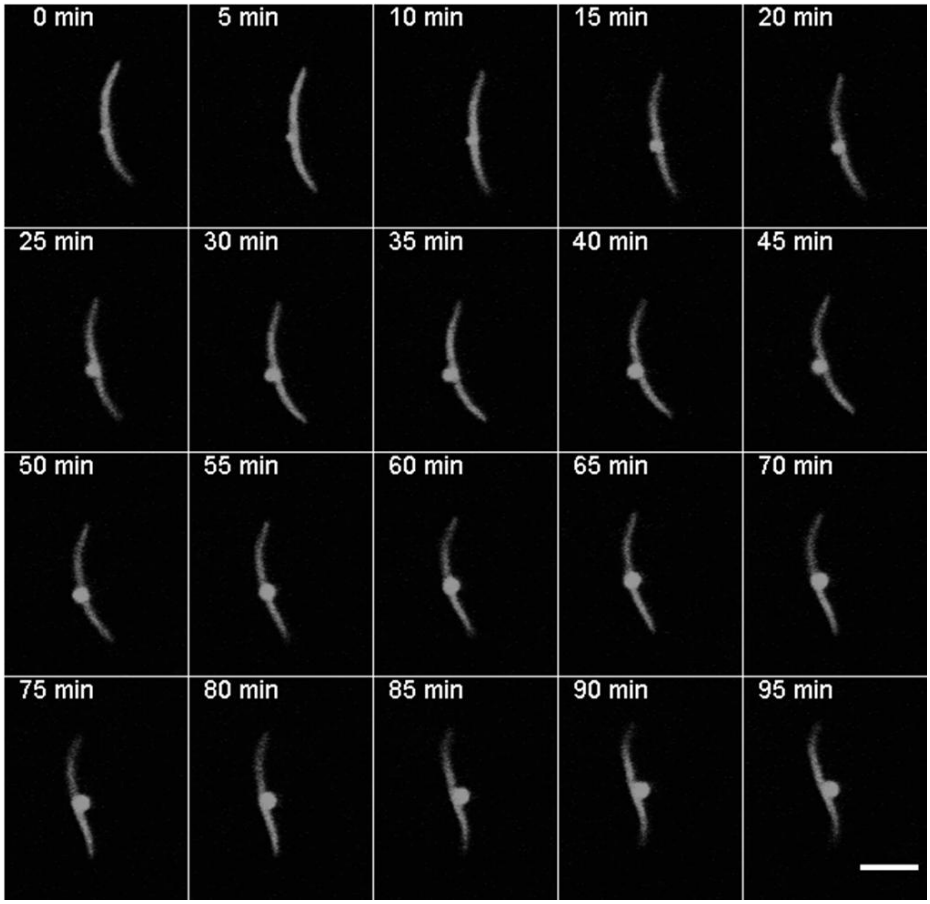


Figure 2.3 – P.berghei dynamic morphological modifications in Hepa1-6 cells. Time lapse frames showing a live GFP-*P.berghei* parasite within a Hepa1-6 cell. Cells were infected and imaged directly using an inverted SP5 Leica resonance confocal microscope equipped with a controlled CO_2 and humidity chamber. Images shown were taken every 5 minutes and span a total of one hour and 40 minutes. Scale bar: 5μm.

It is important to note that *Plasmodium* liver development is dramatically fast, where from one single parasite, an estimated 10-30,000 merozoites are formed in less than 70 hours in one single exo-erythrocytic form (EEF) (Prudêncio et al., 2006). Indeed, when the size of EEFs is measured by measuring the area occupied by a single EEF, and plotted over time, as seen in **Figure 2.4**, two different developmental stages may be observed. An initial stage, from infection to 16-18 hours post infection, where parasites transform from the elongated sporozoite form into the round schizont form and where

size increase is not very significant. After this transformation, a late stage of infection, parasite growth and replication occurs at an astonishing rate until full merozoite formation occurs.

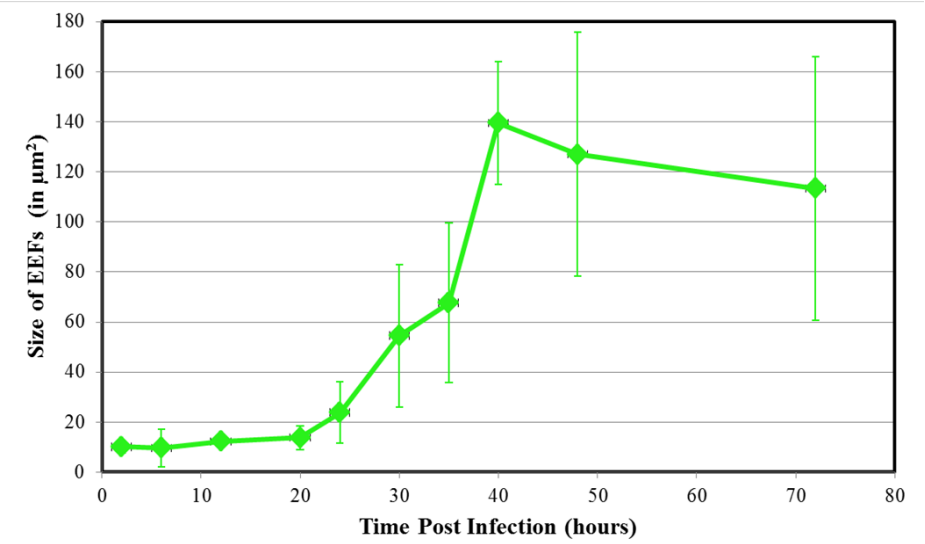


Figure 2.4 – P.berghei EEF size increase in Hepa1-6 cells. Hepa1-6 cells were infected with *P.berghei* parasites and infection was stopped at various hours post infection (x-axis). Parasites were stained with 2E6 antibody and EEF size was quantified by measuring the cytoplasmic area using ImageJ software, in μm^2. At least 30-40 parasites were measured for each time point shown. Mean size with SD is shown for each time point.

With such a dramatic morphological change and size increase, one of the highest eukaryotic replication rates in the world (Sinnis and Sim, 1997), it seems reasonable to assume that parasites require an abundant supply of nutrients and proteins, especially during the late stages of liver infection. As an obligate intracellular pathogen during this stage of its life cycle, the main possible source of nutrients is the host liver cell. This prompted the question of how this nutrient acquisition is achieved and what are the host-parasite interactions required for this to happen, while at the same time, avoiding host cell mediated death.

In order to analyze this interaction in more detail, transmission electron microscopy studies were performed. Initial images show that a variety of host

organelles were found in close proximity to *P.berghei* parasites in Hepa1-6 cells at 24 hours post infection (**Figure 2.5**). Interestingly, various circular membrane bound vesicles are seen very close to the parasite PVM. This suggested that an interaction with some of these host organelles could be happening which was further investigated.

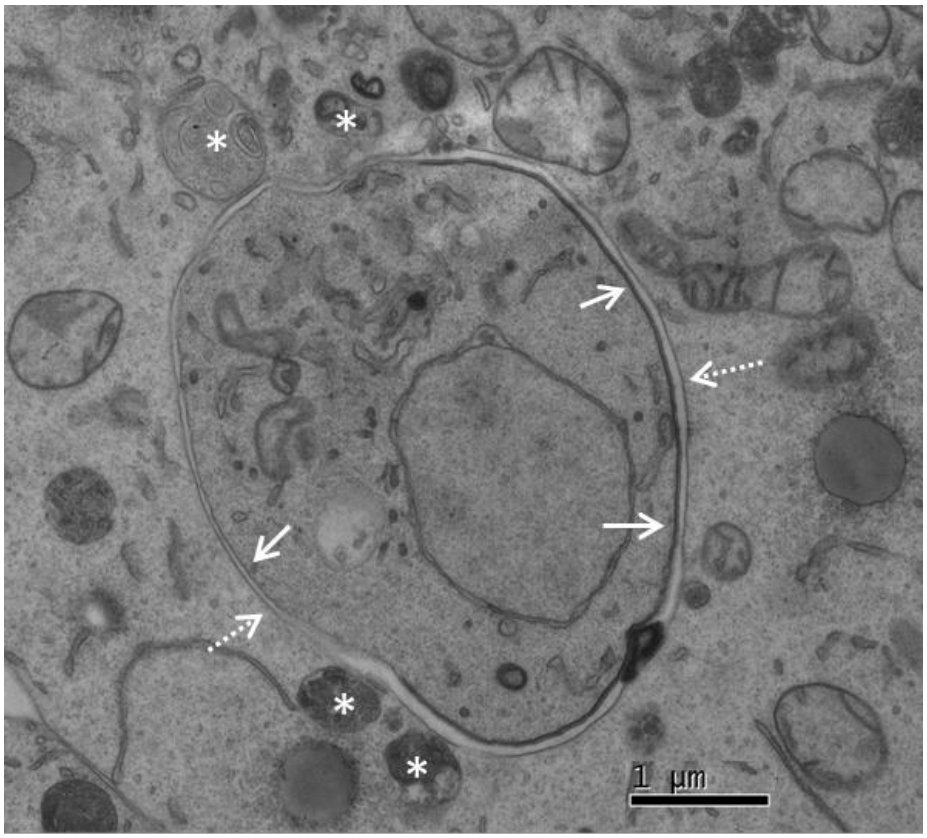


Figure 2.5 – Transmission Electron Microscopy image of a P.berghei parasite 24 hours post infection suggests possible parasite-host vesicle interactions. Transmission electron microscopy samples were prepared of 24 hour infected Hepa1-6 cells. Solid arrows point to the distinct electron dense parasite membrane. Dashed arrows indicate the PVM. Multiple vesicles are seen around the parasite (*). Scale bar: 1µm.

2.2 Relationship between *Plasmodium* parasites and host intracellular organelles

Once inside the hepatocyte, *Plasmodium* parasites encounter a multitude of host organelles and proteins. As mentioned in the introduction, some host-parasite interactions have already been studied, but we were more interested in looking at interactions with the endomembrane system of the host.

2.2.1 Interactions with host ER, Golgi apparatus and peroxisomes

The only host organelle which has been described to interact in some way with the growing *Plasmodium* parasites inside the liver is the host Endoplasmic Reticulum (Bano et al., 2007). Thus we proceeded to analyze this and other host membranes and vesicles in order to investigate any other possible host-parasite interaction during this stage of the *Plasmodium* life cycle.

The Endoplasmic Reticulum (ER) is an extensive membrane network of cisternae, tubules and vesicles, continuous with the perinuclear space, that are held together by the cell cytoskeleton. There are at least three varieties of ER; the rough ER where proteins are synthesized; the smooth ER which serves various functions namely the site where lipids and steroids are synthesized; and the sarcoplasmic reticulum where calcium levels are regulated. In the lumen of the ER, molecular chaperones facilitate proper protein folding and transport of synthesized proteins to the Golgi complex (English et al., 2009).

To investigate the localization of the host ER during *Plasmodium* liver infection, Hepa1-6 cells where infected and infection was stopped at various times post infection. Cells were fixed and stained with anti-Calnexin antibody to mark the host ER, and 2E6 antibody, to stain the parasite cytoplasm.

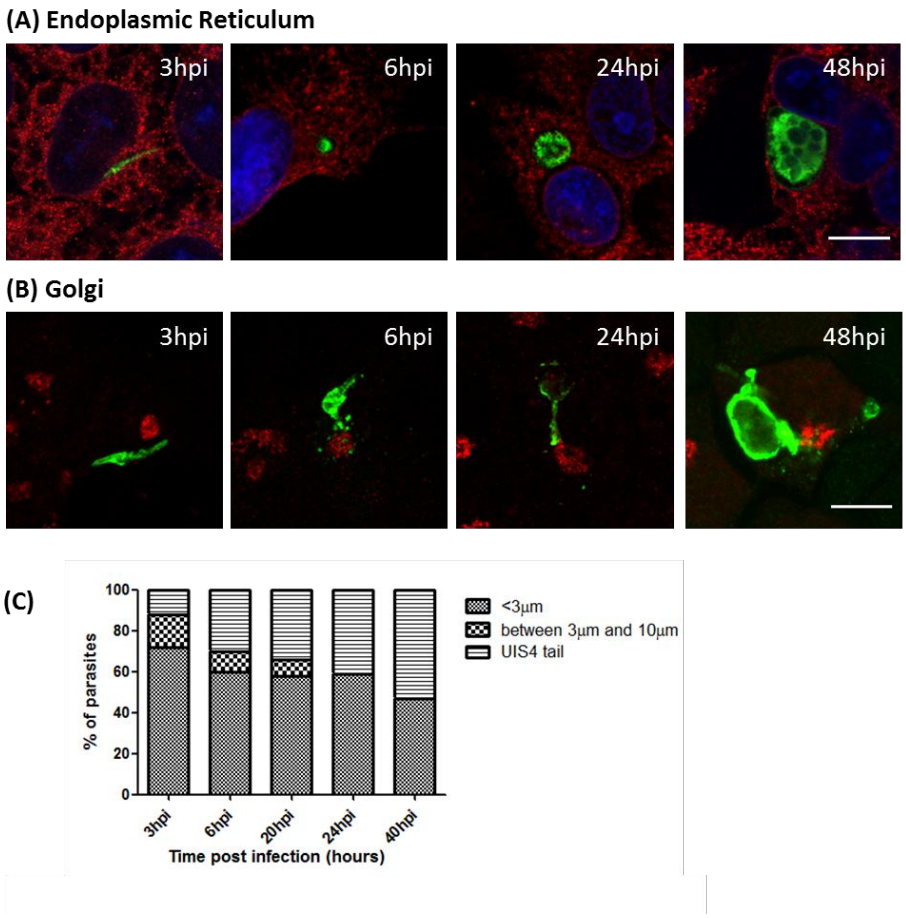


Figure 2.6 – Host endoplasmic reticulum and Golgi during P.berghei liver infection. Hepa1-6 cells were infected with *P.berghei* sporozoites and infection was stopped at various time post infection. (A) Host Endoplasmic Reticulum, stained with anti-Calnexin antibody (red) and parasite cytoplasm was stained with 2E6 antibody (green). (B) host Golgi apparatus, marked by transducing Hepa1-6 cells with GFP-OSBP (red) and parasite PVM was stained with anti-UIS4 antibody (green). Scale bar: 10µm. (C) Quantification of the position of *P.berghei* parasites in relation to the host Golgi. Parasites were divided into categories; parasites found within 3µm from the Golgi; parasites found between 3µm and 10µm from the Golgi; parasites found away from the Golgi but where the PVM extends towards the host Golgi; and parasites with no interaction with the host Golgi (non observed). At least 30 parasites were scored for each time point shown. Results shown as percentage of total parasites counted.

As expected, parasites were seen surrounded by host ER (**Figure 2.6**), although the ER in these cells extended throughout almost all of the host cytoplasm area, which is not surprising since hepatocytes are very metabolically active cells which are constantly producing and metabolizing

large amounts of proteins, molecules and lipids. Surprisingly, a bright rim of fluorescence was only rarely seen around the PVM, as described by Bano et al, (2007), but this could be due to the differences in the cell type used, since Bano et al, used human foreskin fibroblast (HFF) cells, while in this study, a mouse liver cell line was used, a cell type that more closely resembles the biological host cell for this stage of the *Plasmodium* life cycle.

We next investigated any possible association between the Golgi apparatus during *P.berghei* liver infection. The Golgi apparatus is a compact organelle that plays a central role in anterograde transport of newly synthesized proteins from the ER to the plasma membrane or to other intracellular organelles (Sannerud et al., 2003a). Recently, new roles for the Golgi apparatus, such as receiving incoming traffic from endocytic and recycling pathways, cytoskeletal dynamics, organelle biogenesis, glycosylation, receptor signaling and apoptosis have also been shown (Derby and Gleeson, 2007).

In mammalian cells, multiple Golgi stacks (termed *cis*, *medial* and *trans* stacks) are linked together in a ribbon structure around the cell´s centrosome and this ordered configuration must be correctly maintained to ensure correct post-translational modifications of cargo and promote efficient sorting and trafficking of secretory proteins targeted for the plasma membrane (Rios and Bornens, 2003).

A number of protein families are involved in Golgi biogenesis, trafficking and structure maintenance. For example, the golgin family are transmembrane proteins associated with the cytoplasmic face of the Golgi membrane and are recruited in a tightly regulated manner (Gillingham and Munro, 2003). Curiously, *Chlamydia*, an intracellular pathogen, is able to target members of the golgin family to fragment the host Golgi apparatus, facilitating nutrient acquisition (Heuer et al., 2009) and downregulating members of the Rab family of proteins involved in Golgi trafficking decrease *Chlamydia* nutrient acquisition (Rejman Lipinski et al., 2009). From an intracellular pathogen perspective, the Golgi is an ideal organelle for nutrient and protein acquisition.

To analyze the localization of the Golgi apparatus, Hepa1-6 cells were transduced with GFP-OSBP which monitors PI(4)P distribution on the Golgi (Balla et al., 2005). As seen in **Figure 2.6**, *Plasmodium* parasites are often found near the host Golgi network. Quantification of this host-parasite interaction was done by classifying parasites according to their position relative to the host Golgi; parasites found within 3µm from the Golgi; parasites found between 3µm and 10µm from the Golgi; parasites found away from the Golgi but where the PVM extends towards the host Golgi; and parasites with no interaction with the host Golgi.

Strikingly, during the entire liver infection, more than 55% of all parasites were found within 3µm of the host Golgi (**Figure 2.6**). Interestingly, the percentage of parasites found between 3µm and 10µm from the Golgi decreases over time while the percentage of parasites with the PVM extending to the Golgi area increases over time. This close physical association may suggest a possible interaction between *P.berghei* parasites and the host Golgi apparatus. Alternatively, this could be a result of the positioning of the PVM in the perinuclear area, where the Golgi is always found. Although the positioning of the host Golgi near *P.berghei* parasites in the liver has already been described (Bano et al., 2007) the possible role of this organelle during *P.berghei* infection will be further investigated in Chapter 3.

Peroxisomes are single membrane bound organelles present is almost all eukaryotic cells. They are important in the metabolism of fatty acids and many other metabolites as they contain many oxidative enzymes such as catalase, D-amino acid oxidase and uric acid oxidase (Hoepfner et al., 2005). They originate from the endoplasmic reticulum, are proteins carriers within the cell and, most importantly, could be a source of lipids and fatty acids for growing intracellular pathogens. It was recently described that *Plasmodium* liver parasites are able to salvage host cholesterol from the host cell (Labaied et al., 2010) and since peroxisomes are involved in this transport within mammalian cells, we proceeded to analyze the location of these vesicles in infected cells.

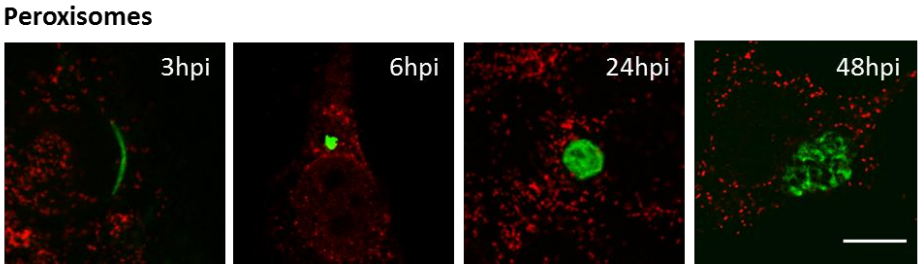


Figure 2.7 - Peroxisomes during P.berghei liver infection. Hepa1-6 cells were infected with *P.berghei* sporozoites and infection was stopped at various time post infection. (a) Cells were stained with anti-PMP70 antibody (red) to stain for peroxisomes and 2E6 antibody (green). (b) Cells were stained with anti-Calnexin (red) to stain for host ER and 2E6 antibody (green). Nuclei were stained with DRAQ5. Scale bar: 10µm.

As seen in **Figure 2.7**, peroxisomes do not seem to interact with the intracellular parasite as they showed no specific aggregation or reorientation around the parasite and show a similar staining pattern as in neighboring non infected cells. Thus there does not seem to be any interaction between these vesicles and *P.berghei* parasites and host peroxisomes.

2.2.2 Interactions with the early and recycling endosome pathway

Early endosomes (EE) are small membrane bound vesicles that are involved in transport of proteins endocytosed at the plasma membrane towards the cell center. The endocytosis of internalized receptors may be processed through numerous routes, but early endosomes serve as focal points of the endocytic pathway (Jovic et al., 2010). Sorting into the various routes occurs at this compartment and proteins and lipids may be either, processed for degradation in lysosomes, delivered to the trans-Golgi network or recycled back to the plasma membrane, on recycling endosomes.

In order to analyze whether early endosomes associate with *P.berghei* parasites during liver infection, Hepa1-6 cells were infected with freshly dissected sporozoites and infection was stopped at various time points after

invasion. Samples stained with anti-Early Endosome Antigen 1 (anti-EEA1) or anti-Transferrin Receptor (anti-TfR) antibody, which stain early endosome and early/recycling endosomes respectively. Rab5 is also a common protein used to mark early endosomes (Zerial and McBride, 2001). As there are no good available antibodies for Rab5, cells were transduced with GFP-Rab5a adenovirus prior to infection with parasites and the localization of Rab5 positive vesicle was analyzed during *Plasmodium* liver infection in cells with a moderate level of Rab5 expression.

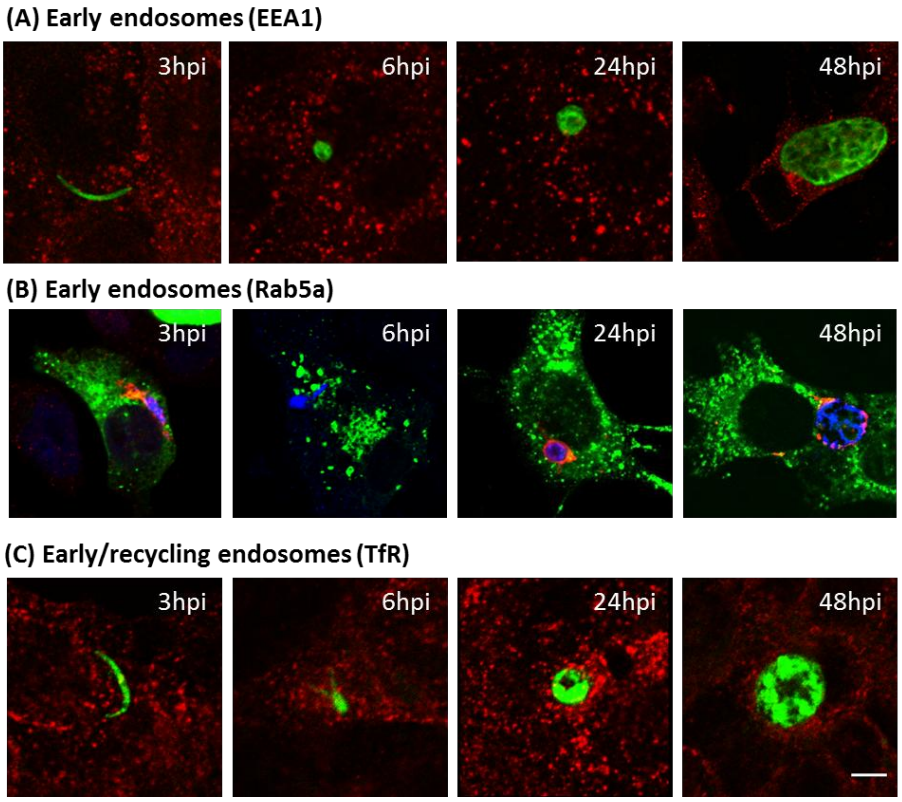


Figure 2.8 – Early and recycling endosome do not aggregate around P.berghei parasite during liver infection. Hepa1-6 cells where infected with *P.berghei* parasites and infection was stopped at various times post infection. (a) Cells were infected and stained with anti-Early Endosome Antigen 1 (anti-EEA1)(red) and 2E6 antibody (green). (b) Cells were transduced with GFP-Rab5 (green) prior to infection with sporozoites and subsequently stained anti-UIS4 (red) and a-2E6 (blue). (c) Cells were infected and stained with anti-Transferrin Receptor (anti-TfR) (red) and 2E6 antibody (green). Scale bar: 10µm.

As seen in **Figure 2.8**, no specific interaction or significant accumulation between the parasite and the host early endocytic and recycling vesicles is seen. No significant reorganization of these vesicles is seen in infected cells compared to non-infected cells, suggesting that the early endocytic pathway does not interact with *P.berghei* parasites during liver infection.

2.2.3 Interactions with the late endosome and lysosomal pathway

Various intracellular pathogens avoid interaction with the late endocytic pathway as it may lead to degradation and pathogen elimination. Nevertheless, other pathogens are able to interact and subvert this pathway to their advantage (see Introduction for a review on this topic). Thus, *Plasmodium* parasite interaction with the host late endocytic and lysosomal pathway was studied.

Rab7 is a member of the Rab GTPase family implicated in transport from the early to late endosomes as well as being involved in controlling the perinuclear aggregation and fusion of late endosomes to lysosomes (Press et al., 1998)(Feng et al., 1995) (reviewed in (Bucci et al., 2000)). Rab7 is one of the better studied members of the Rab family and more recently has been implicated in a multitude of other trafficking events along the endo-lysosome pathway, including the maturation of late autophagic vesicles (Jäger et al., 2004). Interestingly, since it controls a multitude of vesicles in the lysosome maturation pathway, it has been extensively studied in the context of host-parasite interactions, such as in the case of *Salmonella* and *Burkholderia cepacia.*

Rab7 has been widely used as a marker for late endosomes/lysosomes, and was used in this study to investigate for possible interactions between host late endosomes and *P.berghei* parasites. As good antibodies for Rab7 protein

are currently not available, Hepa1-6 cells were transduced with GFP-Rab7a adenovirus and then infected with *P.berghei*.

Lysosome-Associated Membrane Protein 1 and 2 (LAMP1 and LAMP2), and CD63 (also known as LAMP3) are three of the most abundant transmembrane proteins on late endosomes and lysosomes (Huynh et al., 2007). They exhibit considerable sequence homology and have very similar biochemical functions. They have a large heavily glycosylated luminal domain and a short cytosolic tail (Eskelinen et al., 2003), forming a continuous carbohydrate lining on the inner leaflet, producing a glycocalyx. For this reason, they are believed to be responsible for the maintenance of the structural integrity of the lysosome membrane, separating the host cytoplasm from the hostile degradative luminal environment.

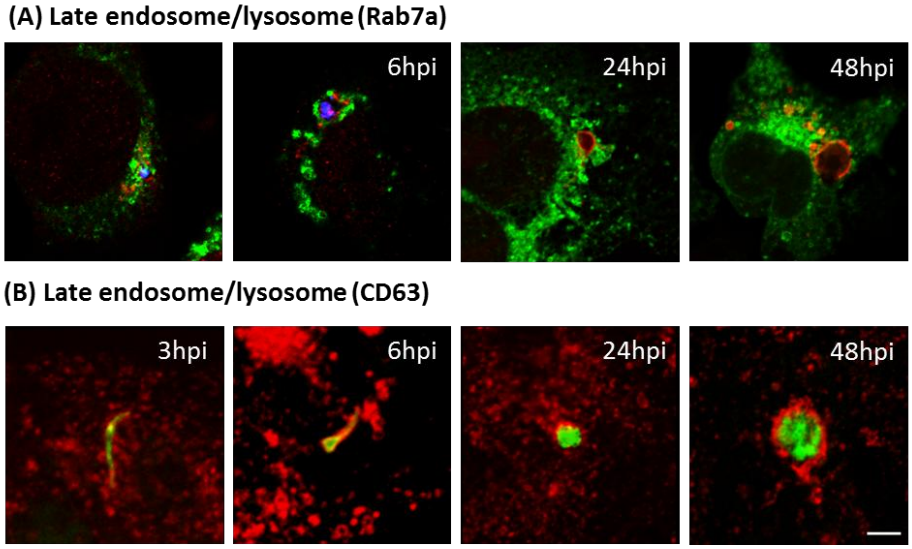


Figure 2.9 – Late endosomes and lysosomes aggregate around P.berghei parasites. (a) Hepa1-6 cells were transduced with GFP-Rab7 (red) prior to infecting with *P.berghei* sporozoites. Cells were fixed at various times post infection and stained with anti-UIS4 antibody (green) to stain the PVM and 2E6 antibody (blue). Nuclei were stained with DRAQ5. (b) Hepa1-6 cells where infected with *P.berghei* sporozoites and infection was stopped at various times post infection. Cells were stained with anti-CD63 antibody (red) and 2E6 antibody (green). Scale bar: 10µm.

As seen in **Figure 2.9** host Rab7a positive vesicles aggregate around the parasite in Hepa1-6 cells. Interestingly, Rab7a vesicles do not seem to cover the entire perimeter of the parasite PVM (although endogenous Rab7a could be more abundant), but show a punctate staining on or near the PVM. CD63, a marker of late endosomes/lysosomes, is also seen to aggregate very strongly around parasites, especially after the initial 3-6 hours of infection. To confirm these results, another more widely used marker of late endosomes/lysosomes, LAMP1 was used to characterize the interaction between these vesicles and the parasite throughout the entire liver infection (**Figure 2.10**).

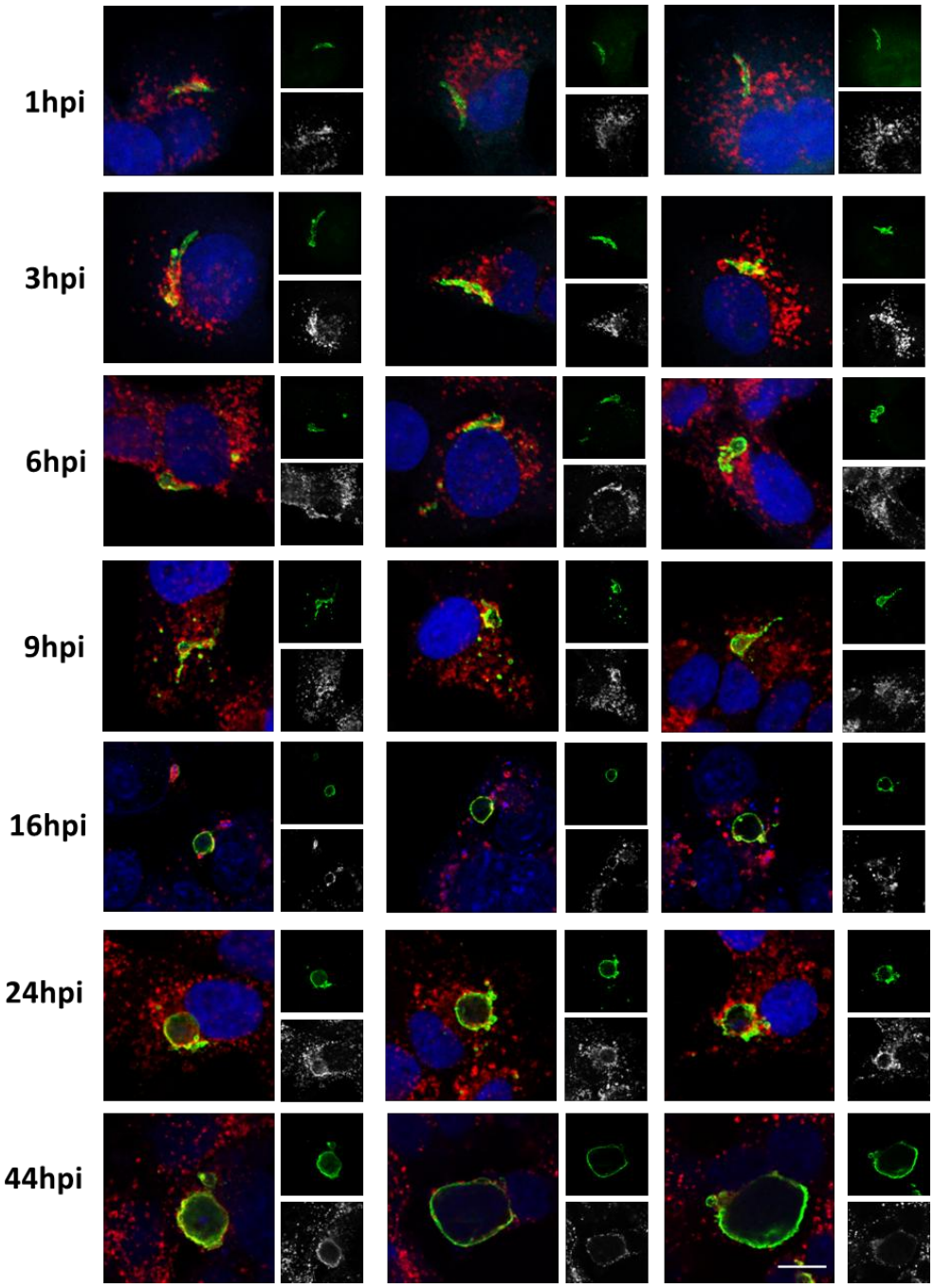


Figure 2.10 – LAMP1 vesicles aggregate around P.berghei parasite throughout the entire liver infection. Hepa1-6 cells were infected with *P.berghei* sporozoites and infection was stopped at various times post infection. Cells were stained with anti-LAMP1 antibody (red) and anti-UIS4 (green) antibody. Nuclei were stained with DRAQ5. Scale bar: 10µm.

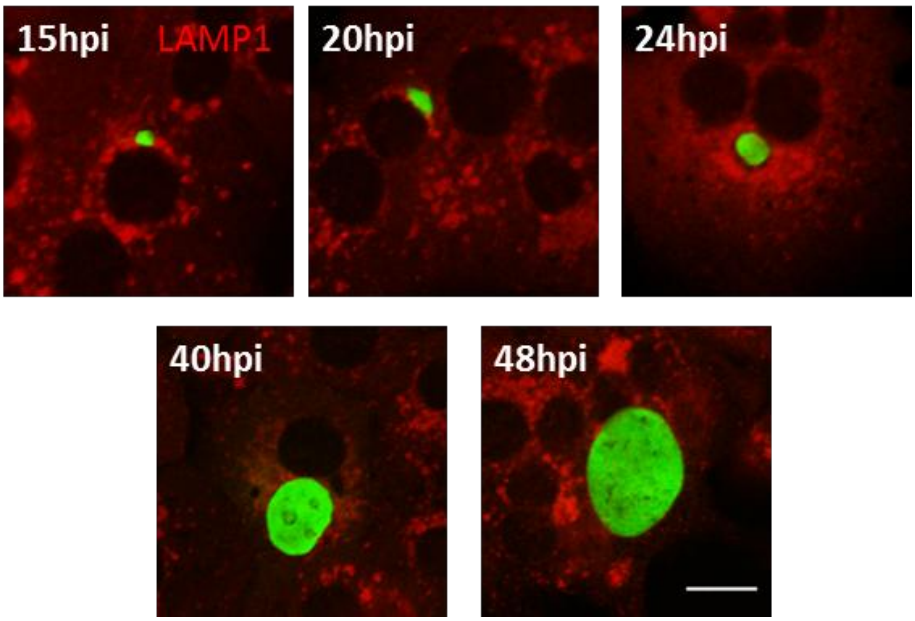
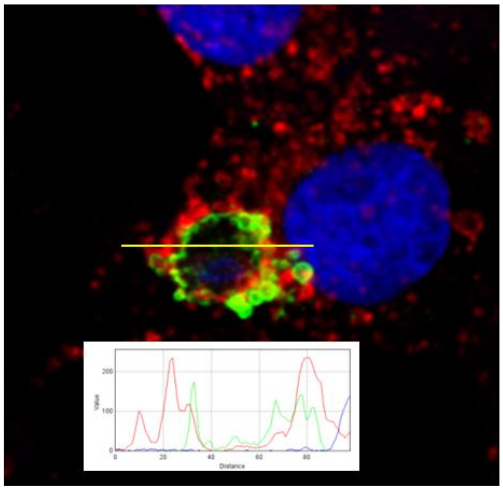


Figure 2.11 – Late endosomes/Lysosomes aggregate around P.berghei parasites in primary mouse hepatocytes. Primary mouse hepatocytes were isolated and infected with *P.berghei* sporozoites. Infection was stopped and cells were stained with anti-LAMP1 (red) and 2E6 (green). Scale bar: 10µm.

As seen with CD63, **Figure 2.10** clearly shows that LAMP1 vesicles aggregate very strongly around the parasite throughout the entire liver infection. The same was seen when using LAMP2 antibodies (not shown) and when mouse primary hepatocyte were isolated and infected, indicating that this process is physiologically relevant and probably occurs *in vivo* (**Figure 2.11**).

In order to visualize and quantify this accumulation, line-intensity plots across parasites were made and analyzed. **Figure 2.12** shows representative line-intensity plots for a parasite at 24 hours post infection. It becomes very clear that there is some degree of colocalization between the PVM (green line) and LAMP1 vesicles (red line) as seen from the two peaks at each side of the parasite PVM profile.

(a)



(b)

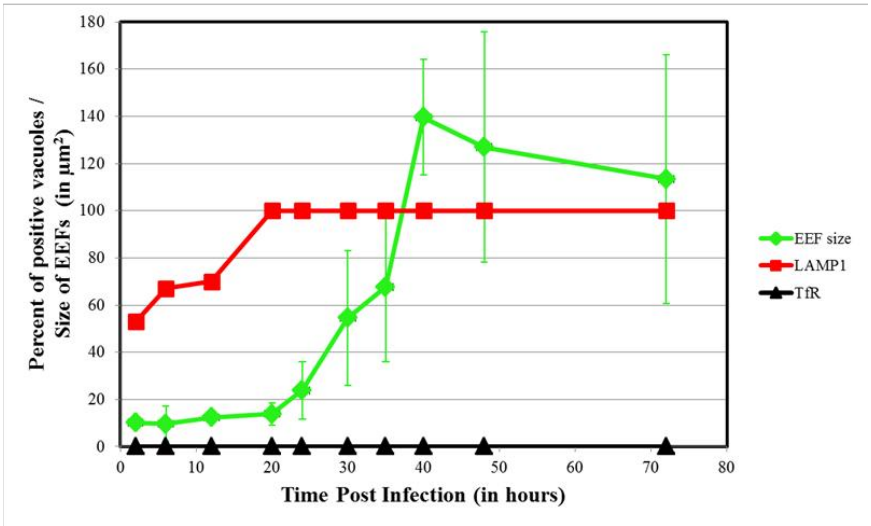


Figure 2.12 – Line plot and kinetics of lysosome aggregation around P.berghei parasites throughout liver infection. (a) Line intensity plot of a 24 hour *P.berghei* parasite PVM stained with anti-UIS4 (green) and LAMP1 vesicles (red) showing a partial colocalization of these two membranes. (b) Quantitative analysis of the kinetics of early endosome (anti-TfR)(black line) and lysosome (anti-LAMP1)(red line) vesicles aggregation around *P.berghei* parasites throughout the liver infection. At least 40 images per time point were analyzed and scored either for positive or negative vesicle aggregation around parasite. Mean size of parasites is also shown (green line) It becomes clear to see that lysosome aggregation occurs long before any significant size increase occurs. Scale bar: 10µm.

This surprising interaction between the host endo/lysosomal pathway and the parasite was quantified, by scoring parasites for either "positive" or "negative" aggregation (**Figure 2.12**). Positive aggregation was scored only when a significant accumulation or a continuous rim of fluorescence was seen around the PVM membrane. The average size of the parasites (as measured in **Figure 2.4**) was plotted on the same graph (green line) to show that LAMP1 (red line) positive vesicle aggregation occurs in 100% of parasites before any significant size increase occurs, and thus is not an artifact of the parasite taking over most of the host cell cytoplasm. As mentioned before, early/recycling endosomes (anti-TfR) were never seen to aggregate around the parasite (black line) at the time points tested.

2.3 Disrupting lysosome function – a gene targeted approach

After the surprising findings obtained in the initial part of this project, where late endosomes/ lysosomes were seen clustering around *Plasmodium* parasites throughout their liver infection, further characterization of this interaction was conducted. Lysosomes are a critical organelle in every cell. Their lumen is filled with highly acidic contents which are separated from the cell cytoplasm by a double membrane that is highly glysocylated. Many acidic hydrolase enzymes are found within lysosomes which in turn are responsible for a variety of key cellular functions, such as digesting lipids, proteins, other macromolecules and phagocytosis material.

Due to the importance of all these functions in the correct maintenance of cellular homeostasis, it is not surprising that lysosome biogenesis, as well as its structural and functional integrity, are all tightly controlled by the cell, using multiple and redundant pathways. Nevertheless, many studies have identified the role of various proteins involved in lysosome biogenesis and control, such as Rab7 (Bucci et al., 2000). Thus, we used some of these described proteins to

try and disrupt lysosome function and investigate its role in *Plasmodium* parasite growth in liver cells.

Rab7 has been shown to be involved in the regulation of the biogenesis and motility of lysosomes from the cell periphery to the perinuclear area. Thus, by disrupting Rab7 function, lysosome motility is impaired (Jordens et al., 2001). We used the same approach to try to disrupt lysosome motility and determine whether this has an effect on *Plasmodium* liver infection.

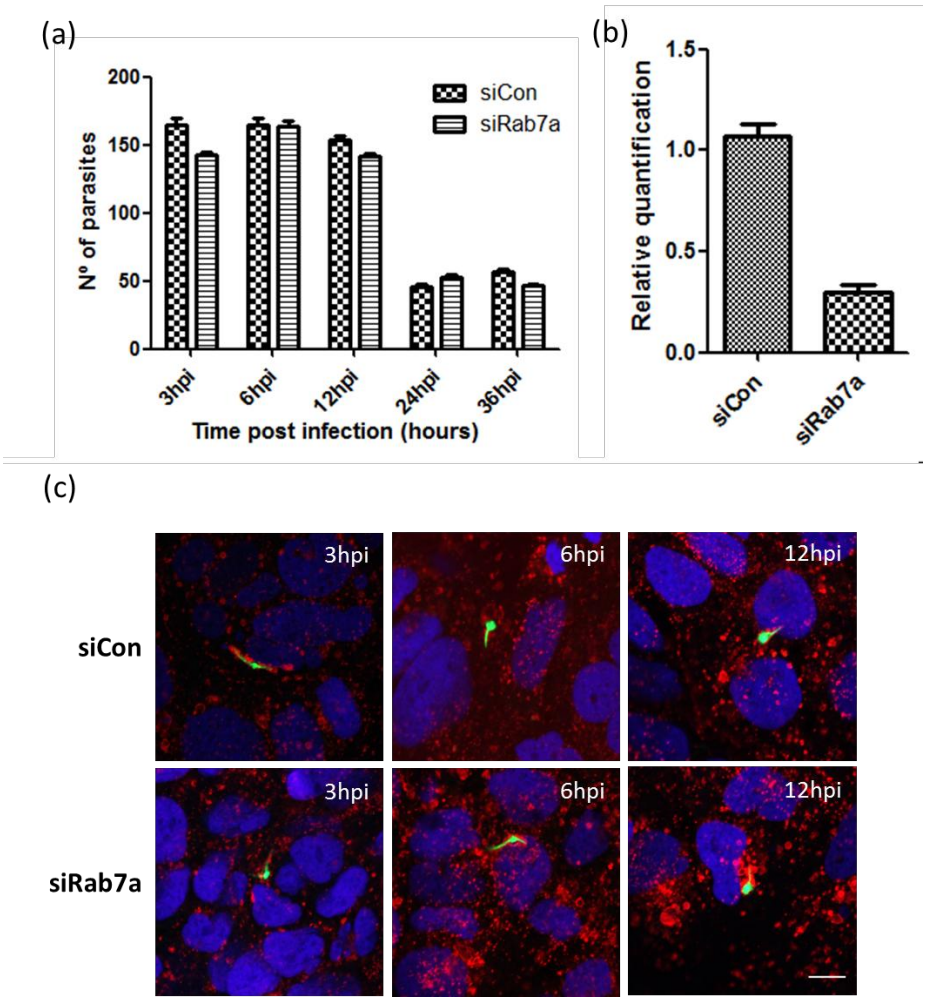


Figure 2.13 – Rab7a knock down has no effect on Plasmodium liver infection. Hepa1-6 cells were treated with siRNA targeting Rab7a and infected with parasites and infected with *P.berghei* parasites 48 hours later. (a) Parasite infection was stopped at various times post infection and total number of parasite was quantified. (b) Relative amount of Rab7a mRNA was measured by qRT-PCR 48 hours post siRNA treatment. (c) siRNA rab7a treated and infected cells were fixed and stained with anti-LAMP1 antibody(red). Parasites were stained with 2E6 antibody, marking the parasite cytoplasm (green). Nuclei were stained with DRAQ5 (blue). Scale bar: 10µm.

Cells treated with siRNA specifically targeting Rab7a were infected with sporozoites and infection rate was quantified at various times post infection. As seen in **Figure 2.13**, decreasing the expression of Rab7 had no effect of *P.berghei* infection rates (and sizes, data not shown) compared to control, for all the time points tested. When siRNA treated cells were stained

with anti-LAMP1 antibody, the staining was equivalent to cells treated with control siRNA (**Figure 2.13**). This would suggest that even though Rab7a mRNA levels were decreased by more than 70% this was not sufficient to impair both lysosome biogenesis and motility, indicating that this is a very tightly regulated process and alternative pathways are probably involved in maintaining lysosome function. Lysosomes were seen aggregating around the parasite in Rab7a siRNA treated cells, just like in control cells, which may explain the lack of effect in parasite infection and development in treated cells.

Further experiments to try to improve the efficiency of mRNA knock down, by treating cells with a second dose of siRNA 48 hours after the first, had no further effect on Rab7a downregulation measured by qRT-PCR (not shown) and no effect on parasite infection. Different concentrations of siRNA were also tested with no success in improving knockdown efficiency.

Although Rab7a downregulation had no effect on lysosome biogenesis and/or motility, other proteins have been described to be involved in this process, including other Rab GTPases, such as Rab9 (Riederer et al., 1994) and Rab32 (on melanosomes, (Wasmeier et al., 2006)). Rab-Interacting Lysosomal Protein (RILP), a Rab7 effector, has also been implicated in lysosome motility (Bucci et al., 2000)(Harrison et al., 2004). Therefore all of these proteins were tested in the context of *Plasmodium* infection in liver cells. (mRNA knock-down levels for each siRNA pool was tested by qRT-PCR where more than 70% of decrease in mRNA level was considered sufficient, data not shown).

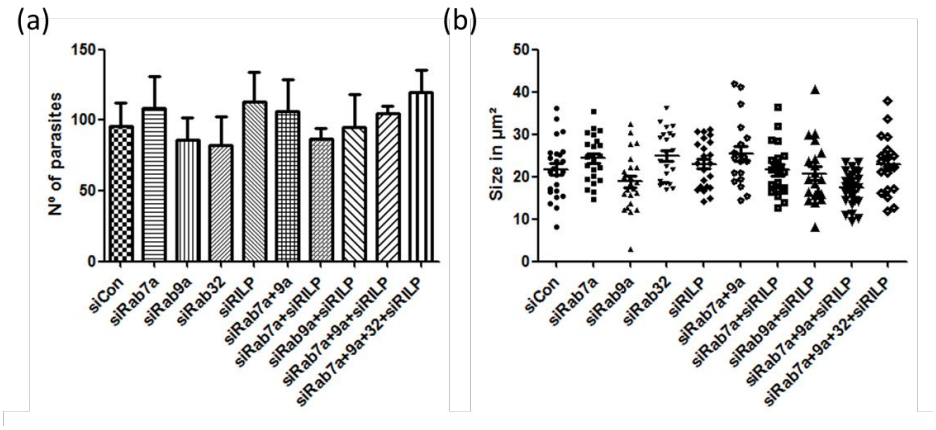


Figure 2.14 – Knock down of proteins involved in lysosome function has no effect on Plasmodium liver development. Hepa1-6 cells were treated with siRNA specific for Rab7a, Rab9a, Rab32 and RILP alone, or in combinations and cells were infected with parasites. Infection was stopped at 24 hours post infection. (a) Total number of parasites per well was quantified and (b) EEF size was measured.

Unexpectedly, treating cells with siRNA specific to these proteins, and even combinations of these proteins, had no effect on both *P.berghei* infection rates and sizes, measured 24 hours post invasion (**Figure 2.14**). Other time points were also analyzed with no significant difference (not shown). Once again, lysosome aggregation around the parasites was never impaired with any of these treatments, which could suggest that either lysosomes are not being sufficiently disrupted with these treatments or that parasites are able to somehow still attract lysosomes to its periphery.

Lysosomes are the primary catabolic compartments of eukaryotic cells. The Lysosomal Membrane proteins LAMP1 and LAMP2 are estimated to contribute about 50% of the proteins on the lysosomal membrane and are also believed to be important for the structural integrity of the lysosome membrane (Eskelinen, 2006). Studies depleting either LAMP1 or LAMP2 have shown that these two proteins share common functions since their individual depletion causes no major phenotypes in mice. However, when both proteins are downregulated together, more severe phenotypes are observed, such as embryonic lethality in mice (Eskelinen et al., 2003) and phagosome maturation arrest in cells (Binker et al., 2007). Thus, we proceeded to treat cells with

siRNA specific to LAMP1 and LAMP2 individually and both together and study their effect during *P.berghei* infection in liver cells.

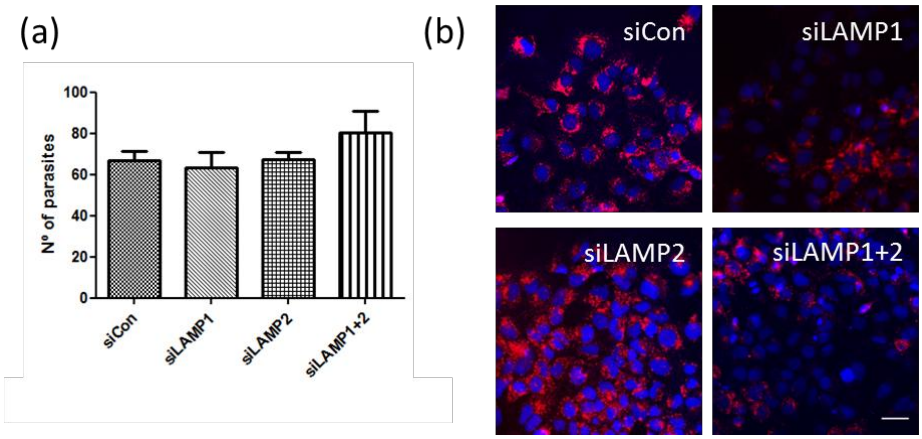


Figure 2.15 – Knock down of LAMP proteins has no effect on Plasmodium liver infection. Hepa1-6 cells were treated with siRNA specific to LAMP1 (siLAMP1)and LAMP2 (siLAMP2) proteins and subsequently infected with *P.berghei* parasites. (a) Parasite infection rate was quantified with no significant difference. (b) siRNA treated (indicated in each image) where fixed 48 hours post siRNA treatment and stained with anti-LAMP1 antibody (red) and DRAQ5 (blue) to stain nuclei. Scale bar:40 µm.

When siRNA treated cells were stained with anti-LAMP1 antibody, there was a decrease in the amount of LAMP1 stained vesicles in LAMP1 depleted cells, indicating that the siRNA was indeed effective. Cells treated with siRNA towards LAMP2 showed normal anti-LAMP1 vesicles, again indicating that, these proteins may compensate for each other. Surprisingly, once again, decreasing both LAMP proteins alone or together seemed to have no significant effect on parasite infection rate measured at 24 hours post infection.

Nevertheless, since all the data suggests that *Plasmodium* parasites are able to subvert their host liver cell in some way, we proceeded to use a more unbiased approach to study this interaction, by performing a siRNA screen of Rab proteins and investigate their effect during parasite liver infection.

2.4 Discussion

During invasion of a hepatocyte cell, *Plasmodium berghei* sporozoites induce the formation of a Parasitophorous Vacuole Membrane (PVM) around them and live within this unique niche. Initial characterization of this stage of the *Plasmodium* life cycle showed that parasites undergo remarkable morphological changes during the first 16-18 hours of liver development. Size of EEFs does not increase dramatically during this phase where parasite replication has not begun. Once this stage has been overcome correctly, and not all parasites survive this stage, parasite replication and growth is initiated. During the next stage of liver development, EEF size increases dramatically and parasites require an abundant amount of nutrients.

Different set of parasite proteins are required for the different stage of liver development which is revealed by microarray data of parasites at different times post infection (Albuquerque et al., 2009). Evidence from different knock-out parasites also suggests the specific role of proteins in earlier or later stages of parasite development in the liver, as some knock-out parasites are able to invade and establish a niche but are not able to proceed to replicate and form late mature liver stages. For example, knock-out parasites for the PVM protein UIS3 are able to establish early infection but are not able to proceed to the later stages, suggesting that the PVM also has an important role in this later stage of development (Mueller et al., 2005).

With such a dramatic size increase and one of the highest eukaryotic replication rates (Sinnis and Sim, 1997), it seems reasonable to assume that parasites require an abundant supply of nutrients and proteins, especially during the late stages of liver infection. Initial electron microscopy images showed that various host vesicles were found in the parasite vicinity, suggesting that host vesicles could be the source of nutrients required by parasite for growth, which we proceeded to further investigate.

Intracellular pathogens, in order to survive and replicate, need to establish an ideal niche to grow and acquire nutrients from the host while at the

same time escape the host´s immune surveillance system. *Plasmodium* parasites are purine auxotrophs and are incapable of synthesizing fatty acids *de novo* (Bano et al., 2007)(Labaied et al., 2010). Hence, the PVM has a dual function since it must protect the parasite from potential harmful substances but also allow, if not facilitate, access to nutrients from the host cell. Thus parasites need to be able to control transport across the PVM and how it interacts with the host cell´s organelles.

Curiously, *Plasmodium* parasites have been shown to move toward the host cell nucleus during liver infection (Bano et al., 2007). This would suggest that parasites are able to interact and exploit the host cytoskeleton for intracellular movement, suggesting again that parasites are able to interact with their host cell environment.

P.berghei parasites were seen surrounded by host ER (**Figure 2.6**), although the ER in these cells extended throughout almost all of the host cytoplasm area, which is not surprising since hepatocytes are very metabolically active cells which are constantly producing and metabolizing large amounts of proteins, molecules and lipids. Surprisingly, a bright rim of fluorescence was only rarely seen around the PVM, as described by Bano et al. (2007), but this could be due to the differences in the cell type used, since Bano *et al*, used human foreskin fibroblast (HFF) cells, while in this study, a mouse liver cell line was used, a cell type that more closely resembles the biological host cell for this stage of the *Plasmodium* life cycle.

Curiously, the host Golgi apparatus was also seen very close to most parasite during the entire liver development. Although this association has already been described (Bano et al., 2007) the role of this interaction is still unknown. It is also possible that the close physical positioning of the Golgi and the parasite is just and indirect effect of the parasite´s perinuclear location, where the Golgi is also located. Interestingly, during the early stages of infection, some parasites are found far away from the host Golgi, with no apparent growth impairment, although in many of these cases, a UIS4 “tail” is seen extending from the parasite PVM to the peri-Golgi area. The possible role

of the host Golgi during Plasmodium liver development will be further characterized in Chapter 3.

When the host endocytic pathway was analyzed, no obvious association between early and recycling endosomes and parasites was seen. Like with other intracellular pathogens, it is possible that such an interacting occurs very transiently, possibly during the first minutes of parasite invasion, although such an analysis is very difficult to perform with *Plasmodium* parasites, due to their ability to migrate before committing to infection, it is very difficult to distinguish invading from migrating parasites during the initial hours of infection. Thus this interaction cannot be discarded at this point.

While very little was known about the composition and origin of the PVM within liver cells, a recent article by Bano et.al (2007) states that "no major alteration in the organization of host lysosomal distribution was observed in *P.berghei* infected cells at 24h post infection," although no data was shown. Nevertheless, and because we believe that some type of host-parasite interaction had to occur, we proceeded to analyze the localization of these vesicles in infected cells.

Surprisingly, when late endosomes/lysosomes were stained in infected cells, a clear aggregation of these vesicles was seen around parasites, throughout the entire liver infection. This was also seen in isolated mouse primary hepatocytes, a cell type that more closely resembles the mouse liver hepatocyte. This aggregation was seen around most parasites during early infection and 100% of parasites during later infection. This surprising result prompted us to question the role of host vesicles during *Plasmodium* liver development, since these vesicles are highly acidic and are part of the cells anti-microbial defense mechanism. Since their fusion with the PVM could lead to parasite degradation and elimination, we proceeded to attempt to disrupt this vesicle to study its role in parasite development.

Unfortunately, and after an exhaustive period of assays using various siRNAs targeting various protein known to be involved in late endosome/lysosome trafficking, none of these treatments had an effect on

parasite infection and development. Unfortunately, none of these treatments seemed to impair late endosome/lysosome integrity in Hepa1-6 cells, seen when cells were stained with anti-LAMP1 antibody. More importantly, these vesicles were always seen surrounding parasites, which could explain the lack of an effect on the parasite.

siRNA has become a very widely used method to downregulate the expression of protein in cells, although it has its disadvantages, namely, for long lived proteins such a Rab proteins, a decrease in mean mRNA level of 70% is generally considered a good knock-down. From the images in **Figure 2.15**, where cells were treated with siRNA towards LAMP1 and then stained with anti-LAMP1 antibody, many cells were completely negative for LAMP1 while others had visually normal LAMP1 vesicles, suggesting that siRNA treatment is very effective in the cells where it is internalized but this does not occur in 100% of the cells. This makes it extremely difficult to use efficiently, especially when using a pathogen that has a 3-5% infection rate and where the probability of finding a parasite within a siRNA treated cell is low. Nevertheless, since all the data suggests that *Plasmodium* parasites are able to subvert their host liver cell in some way, and taking a gene targeted approach to disrupt this interaction was unfruitful, we proceeded to a more unbiased approach, performing a siRNA screen of Rab proteins and investigate their effect during parasite liver infection.

3. siRNA Screen of host trafficking proteins and their role in *P.berghei* liver stage infection

All the work presented in this chapter was performed by Mafalda Lopes da Silva with the help of Alistair Hume, Imperial College London, with the exception of the production of the virus constructs used which was done in collaboration with José Ramalho, FCM, and the experiment shown in Figure 3.10 which were performed and provided by Laura Santos.

Summary

The previous chapter showed that *Plasmodium* liver parasites are found surrounded by late endosomes/ lysosomes, suggesting that parasites are able to interact with the host endomembrane system. Using a library of siRNA directed against the Rab GTPases family, the effect of down regulating these proteins was studied in the context of malarial infection. Only one siRNA treatment had a significant effect on parasite liver infection rate and this was, surprisingly, Rab1a. Using a library of GFP-tagged Rab protein constructs, colocalization experiments of GFP-Rab proteins and liver stage parasites was performed. Parasites were often found near or surrounded by a number of Rabs, and interestingly, most of these have been proposed to be involved in some way in Golgi or ER trafficking, suggesting a possible role of this host organelle in *Plasmodium* liver development.

The functional hit obtained in the siRNA screen was further characterized and its role during *Plasmodium* liver was investigated. Rab1a has been implicated in ER-to-Golgi trafficking and more recently, in autophagy, more specifically, in initial autophagosome formation. Unexpectedly, Rab1a seems to be affecting *Plasmodium* liver parasites through its role in autophagy and not through the ER-to-Golgi trafficking pathway. More importantly, Rab1a seems to be important during the initial stages of parasite development by enhancing parasite killing, since its absence leads to an increase in parasite infection rate. During the later stages of infection, Rab1a seems to be important for parasite replication, since EEFs are smaller in Rab1a depleted cells. Colocalization experiments with the autophagic marker LC3 showed that LC3 vesicles aggregate very strongly around parasites and seem to be upregulated in infected cells suggesting a new and important role for autophagy during *Plasmodium* liver infection.

Materials & Methods

Cell lines, Parasite strains, microscopy sample preparation, virus production siRNA transfection and qRT-PCR

All performed as described in Chapter 2.

For samples where the anti-LC3 antibody was used, cells were fixed with ice cold methanol for 5 minutes, and subsequently processed as described in Chapter 2.

Antibodies

Apart from the antibodies already described in Chapter 2, rabbit polyclonal anti-LC3 antibody (Sigma; 1:2500) and anti-GFP (Molecular Probes, Invitrogen; 1:100). Secondary antibodies (Alexa) were from Invitrogen and used at 1:400.

Virus

Viruses used in this chapter: GFP-Rab1a, GFP-Rab1b, GFP-Rab2b, GFP-Rab3a, GFP-Rab6a, GFP-Rab6b, GFP-Rab8a, GFP-Rab8b, GFP-Rab9a, GFP-11a, GFP-Rab11b, GFP-Rab14, GFP-Rab15, GFP-Rab20, GFP-Rab21, GFP-Rab22, GFP-Rab29, GFP-Rab31, GFP-Rab33b and GFP-Rab43 (see Supplementary Table 2 for details).

qRT-PCR primers

The following primers were used in this Chapter for qRT-PCR: Mouse Rab1a primers (sense, 5′- atgtgacagatcaggagtcc -3′, and antisense, 5′- tgactgcttgactggagtgc -3′), mouse Rab1b primers (sense, 5′- actgaccaggagtcctacgc -3′, and antisense, 5′- taccagcagccaccgctagc -3′), mouse

Rab3a primers (sense, 5´- ttaatgcagtgcaggactgg -3´, and antisense, 5´- caggcacaatcctgatgagg -3´), mouse Rab8a primers (sense, 5´- agtcctttgacaacatccgg -3´, and antisense, 5´- actgcaccggaagaagctgg -3´), mouse Rab11a primers (sense, 5´- cgatggctgaaagaactgag -3´, and antisense, 5´- gacagcactgcacctttggc -3´), mouse α-Tubulin primers (sense, 5´– ggtggatctagaacct-3´ and antisense, 5´– cccagtgagtgggtcagc-3´). P.berghei ANKA 18s primers (sense, 5´– ggagattggttttgacgtttat-3´ and antisense, 5´– aagcattaaataaagcgaata-3´).

siRNA screen

Hepa1-6 cells ($5x10^3$ per well) were seeded in optical 96-well plates (Costar). 24 hours later, 100nM of gene specific siRNA pools (Dharmacon) were diluted in 20µl of Optimem (Gibco, Invitrogen) while 0.4µl of Oligofectamine (Invitrogen) were added to 30µl of Optimem. These mixtures were combined and incubated at room temperature for 20 minutes. Medium from cells was removed and the siRNA mixture was added to each well. After 3 hours of incubation at 37ºC, cells were washed and normal medium was added. Each siRNA depletion was performed in triplicate. See Supplementary Table 1 for a list of genes tested and siRNA sequences.

Cell were infected with freshly dissected *GFP-P.berghei* ($2x10^4$ per well) and infection was stopped after 24 hours by washing with PBS and fixing with 4% PFA for 15 minutes. Samples were permeabilized with 1% BSA (Sigma), 0.05% saponin (Sigma) and 1% FCS (Gibco, Invitrogen). Parasites were detected using an anti-GFP antibody (Molecular probes, Invitrogen) and a Goat anti-rabbit-Alexa488 secondary antibody (Molecular Probes Invitrogen). Nuclei were stained with DAPI (Invitrogen). Samples were maintained in PBS at 4ºC until ready for image acquisition.

Automated image acquisition and analysis

Images were acquired using the Cellomics HCS system (ThermoScientific), using a 10x lens. A total of 25 images per well were taken for both the GFP channel and the DAPI channel. Image data was analyzed using a custom ImageJ plugin, where parasites and nuclei were identified and counted automatically. Total number of parasites and nuclei per well was obtained and infection rate was calculated as the number of parasites divided by the number of nuclei in each well, normalized to the median of all control wells in each plate. Z-score for each well was calculated by subtracting the mean of the controls wells of each plate from the infection rate value, and dividing this by the mean Standard Deviation of the control wells.

Parasite migration assay

To measure parasite migration through cells prior to invasion, Hepa1-6 cells ($7x10^3$ per well) on a 96-well plate were incubated for 45 minutes with 10μM of Calcein Green AM (Molecular Probes/Invitrogen) diluted in HBSS medium (Gibco/Invitrogen). Calcein Green enters cells and is retained by the cell membrane. When parasites migrate, the host cell membrane is disrupted allowing release of the internalizes Calcein into the medium. Thus, prior to infection with 100μl of growth medium with *P.berghei* sporozoites ($1x10^4$), cells were washed with growth medium to remove all Calcein from the extracellular medium. Cells were infected and the supernatant was gently collected one hours post infection and its fluorescence was measured using a spectrophotometer (excitation: 485nm; emission: 535nm).

3.1 siRNA screen development and results

The previous data presented in this thesis strongly suggests that *Plasmodium berghei* parasites are able to manipulate the host liver cell. Furthermore, parasites require a significant quantity of nutrients and lipids to grow and replicate, which they most likely obtain from the host liver cell. Since a gene targeted approach, towards proteins known to be involved in lysosome traffic did not yield any conclusive results on the role of late endosomes/lysosomes in *Plasmodium* infection, we reasoned that a more unbiased approach could give us clues as to the molecular players involved in this host manipulation by the parasite. Thus, we took advantage of a small siRNA library covering the majority of the Rab GTPases family to study their role during parasite growth and development.

siRNA technology has been widely used to study host-parasite interactions (Prudêncio and Lehmann, 2009). During the past years, various groups have used this technique to identify host proteins involved in intracellular pathogen survival and replication. Studies where siRNA screens were employed to study host-pathogen interactions, such as studies using *Listeria* (Cheng et al., 2005)(Agaisse et al., 2005), *Brucella* (Qin et al., 2008), *Fransicella* (Akimana et al., 2010) and *Mycobacterium* species (Philips et al., 2005) have been critical at identifying new host proteins involved in pathogen infection. siRNA screen studies using virus infections such as Hepatitis C virus (HCV) (Tai et al., 2009) and Human immune deficiency virus (HIV) replication (Zhou et al., 2008) have also uncovered the roles of new proteins in virus infections. Curiously, in many of these genome wide screens, Rab proteins are often significant hits, indicating that this family of proteins is crucial for maintenance of adequate phagosome integrity and are often targeted by pathogens for host manipulation and survival.

At the time this study was initiated, the use of siRNA technology to study parasite-host interactions was very limited. A few studies have attempted to find new host proteins involved in Plasmodium development within the

mosquito host (Brandt et al., 2008). Studies in the mammalian host have also been developed for the liver stage of Plasmodium life cycle (Rodrigues et al., 2008)(Prudêncio et al., 2008). These types of studies have utilized new and important tools to evaluate the importance of host proteins during parasite infections and could lead to new therapies.

In the current study, short interfering RNA oligonucleotides (siRNAs) were used to treat Hepa1-6 cells and down-regulate the expression of individual murine Rab proteins. The quantities of siRNA used were optimized for a number of Rabs and these conditions were applied to the remaining targets. Rab GTPases are known to be very difficult to downregulate, and so, for each gene, a pool of 4 individual oligonucleotide sequences was used to maximize knock-down levels. A 60% reduction in total protein expression in these cells was considered to be, on average, a good knock-down (**Figure 3.1**). After optimization, 100uM of each siRNA pool and an incubation time of 48 hours before infection with sporozoites were used throughout the rest of the experiments shown.

In total, 50 genes were tested in this screen, in triplicate. Hepa1-6 cells were seeded in 96-well plates (Corning) and transfected with siRNAs the next day. 48 hours after siRNA transfection, freshly dissected GFP-*P.bergei* sporozoites were added to the cells. Infection was stopped by fixing cells 24 hours post infection and stained for parasites, using anti-GFP antibody, and nuclei were stained with DAPI. (see **Figure 3.1** for a flow diagram of the protocol followed). Image acquisition was performed automatically and a total of 25 images were acquired per well. Image analysis was performed automatically using a custom-made ImageJ plugin (see Materials & Methods). Total number of parasites for each well and total number of nuclei were quantified automatically and infection rate (total number of parasites divided by total number of nuclei) was calculated for each individual well.

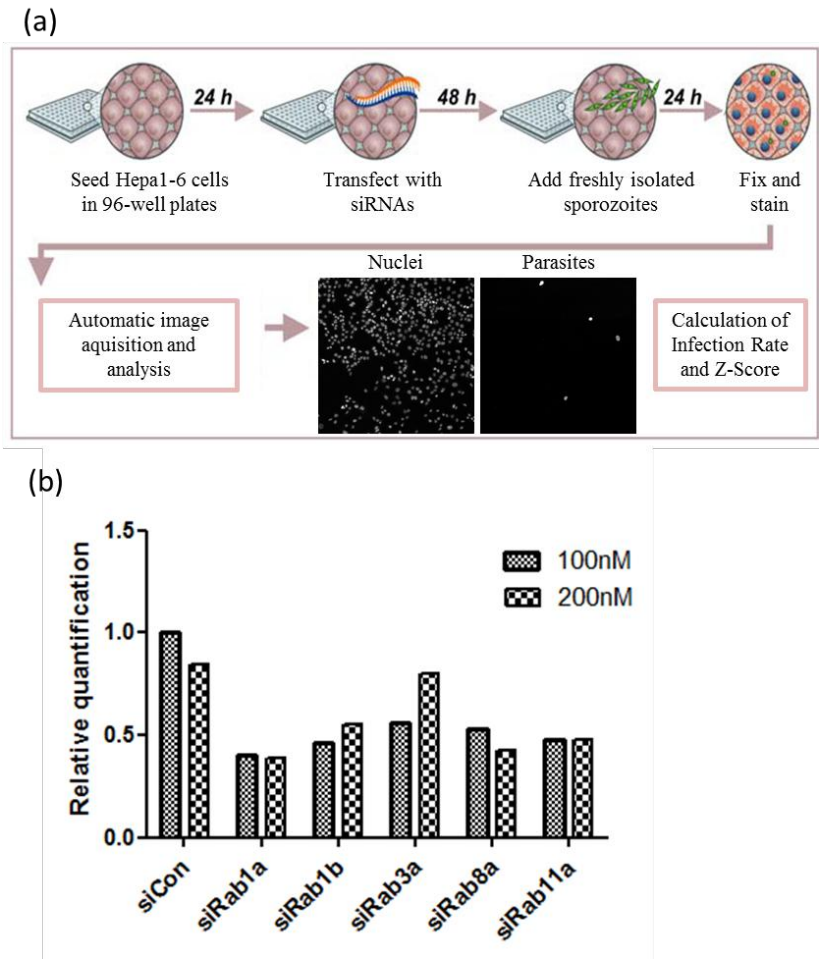


Figure 3.1 - siRNA screen strategy to identify host factors involved in Plasmodium liver infection. (a) Experimental design of siRNA screen. (b) Optimization of siRNA amounts used by evaluating the knock-down efficiency of 5 genes included in the screen.

Individual well infection rates were standardized by comparing them with the mean infection rates of all the controls (treated with control non-targeting siRNA) on the same plate, and the mean of the triplicates was calculated for each gene as well as the Standard Deviation. These results are shown in **Figure 3.2**. The z-score for each sample was also calculated and used as a measure of significance of the results (see (Ramadan et al., 2007) for a very good review on siRNA screen analysis).

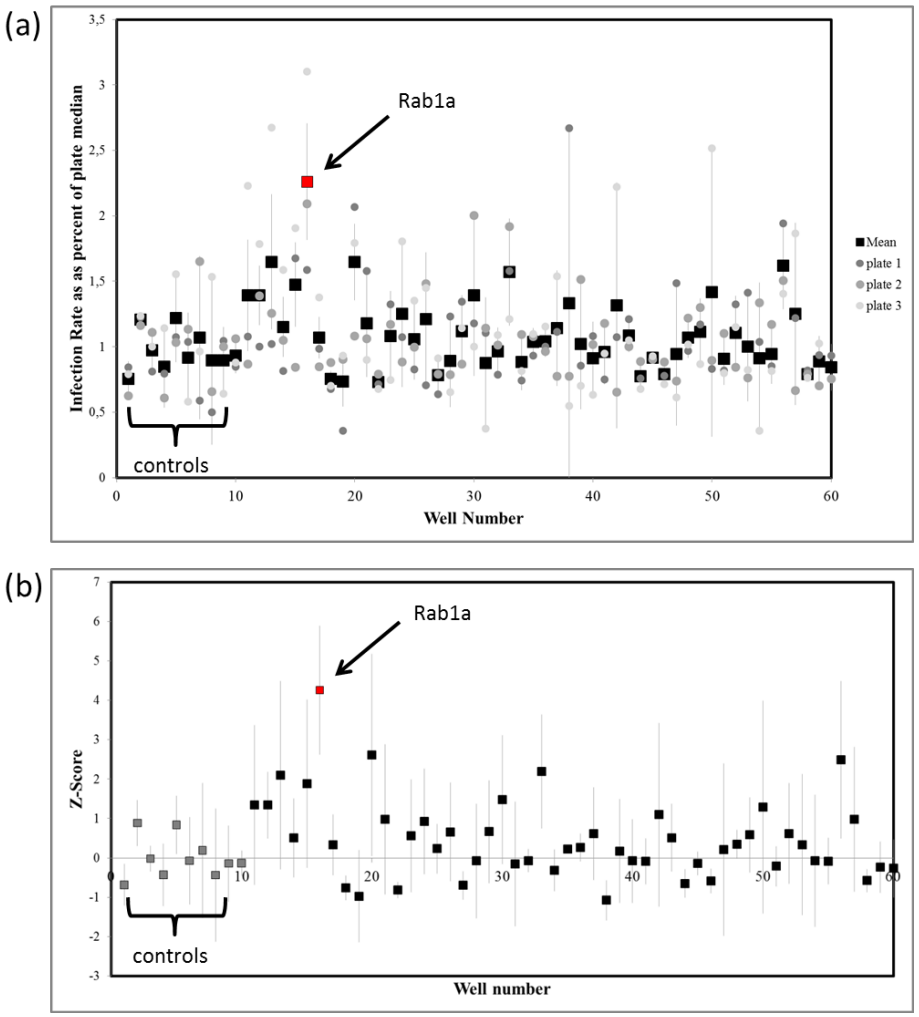


Figure 3.2 – Results of siRNA screen of effect of host Rab proteins during Plasmodium liver infection. Hepa1-6 cells were treated with siRNA pools targeting Rab GTPases and infected with GFP-*P-berghei* sporozoites 48 hours later. Cells were fixed 24 hours later, parasites were stained with anti-GFP antibody and nuclei with DAPI. (a) Mean (square) and individual (circles) *P.berghei* infection rates, relative to the mean of control wells, with Standard Deviation (lines). (b) Mean Z-Scores for each condition (square), relative to mean of control wells, with Standard Deviation (lines). Results for Rab1a are shown in red.

As shown in **Figure 3.2** (and **Supplementary Table 3**), where samples 1-10 are control wells, most siRNA pools had no significant effect on parasite infection rate when measured after 24 hours of infection. Interestingly, siRab7a

as well as siRab9a, both already previously tested, showed no effect on parasite growth, validating the current results. The only significant hit was well 17, which was treated with a siRNA pool specific for murine Rab1a. This unexpected result was validated and further characterized.

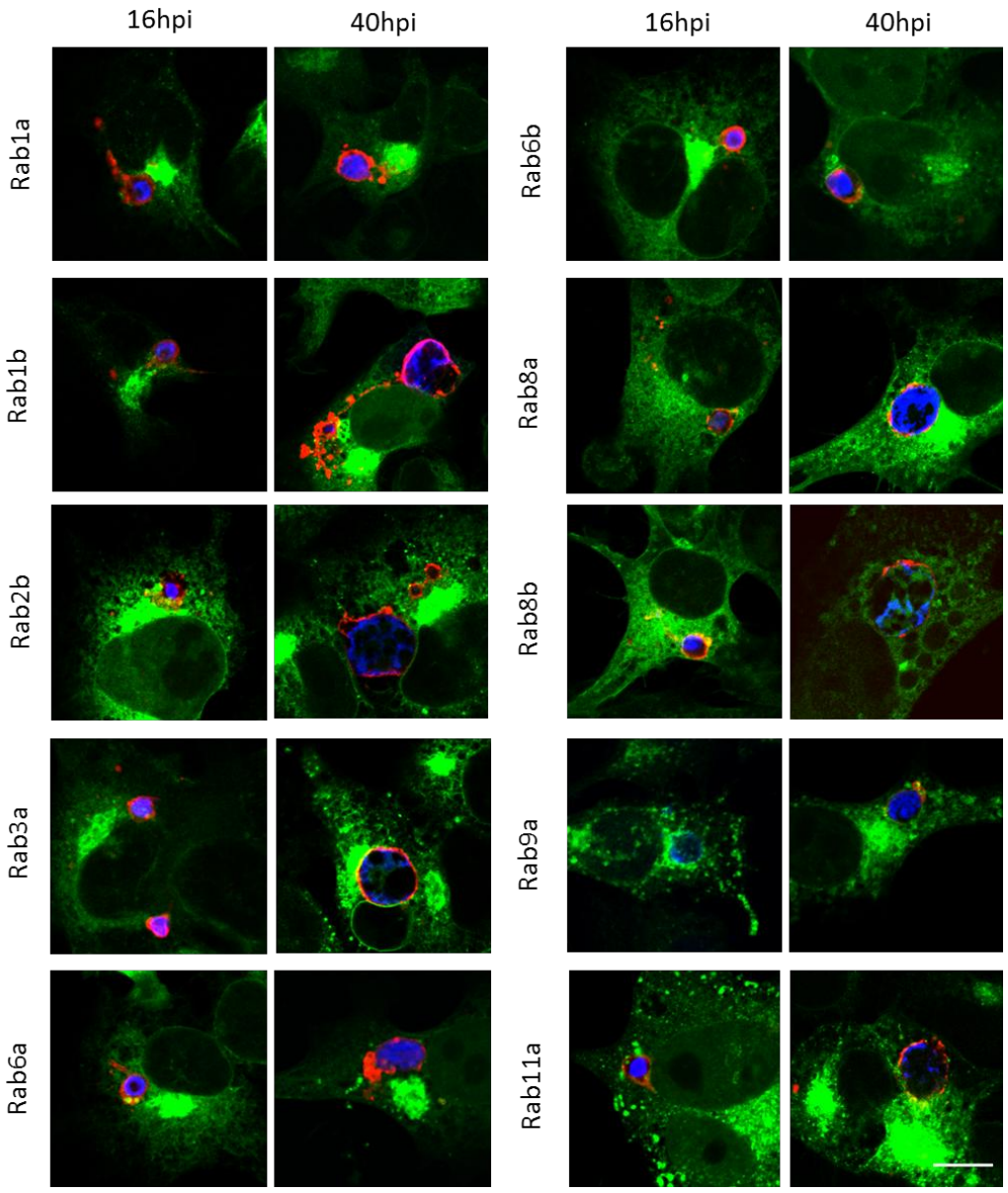
It should be noted at this point that the present data does not fully exclude the involvement of other Rabs amongst those tested in *Plasmoudium berghei* infection in the liver, since negative results in siRNA screens are generally inconclusive (Echeverri and Perrimon, 2006). Furthermore, only one time point after infection was tested (24 hours) and only infection rate was measured as size differences at this point would be too unreliable to measure in a high-throughput context. Thus, there could be proteins amongst those tested involved in a later stage in infection and/or size growth, which would not be identified in this study. Moreover, mammalian Rab GTPases are known to be long lived and display functional redundancy within sub-families (Barral et al., 2002). Hence, depletion of one Rab isoform can be quickly compensated by the overproduction of another isoform (Mizuno et al., 2007). Thus it is very difficult not only to down-regulate Rab proteins using siRNA technology but also to completely target a specific Rab function, due to the functional redundancy in this family of proteins.

3.2 Plasmodium colocalization with Rab proteins

Concurrently with the siRNA screen, the colocalization of a number of Rab proteins with parasites was investigated as has been performed for other intracellular pathogens such as *Anaplasma* (Huang et al., 2010), *Chlamydia* (Rzomp et al., 2003) and more recently, *Mycobacteria* (Seto et al., 2011). Taking advantage of a library of GFP-Rab constructs packaged within an adenovirus infection system (see Materials & Methods), the transduction rates were maximized and the probability of finding a parasite within a GFP expressing cells increased dramatically, compared with normal transfection

methods. Twenty murine Rab proteins were analyzed in terms of their location in relation to *Plasmodium* parasites during liver development.

Each GFP-Rab protein was tested at two different times during Plasmodium infection; 16 and 40 hours post invasion, in order to get an early and a late stages of the parasite development in the liver. **Figure 3.3** shows Hepa1-6 cells expressing GFP-Rab proteins (in green), the parasite cytoplasm stained using 2E6 antibody (in blue) and the parasite PVM, when shown, was stained using anti-UIS4 antibody (in red).



Figures 3.3– GFP-Rab localization during P.berghei liver infection. Continues in next page.

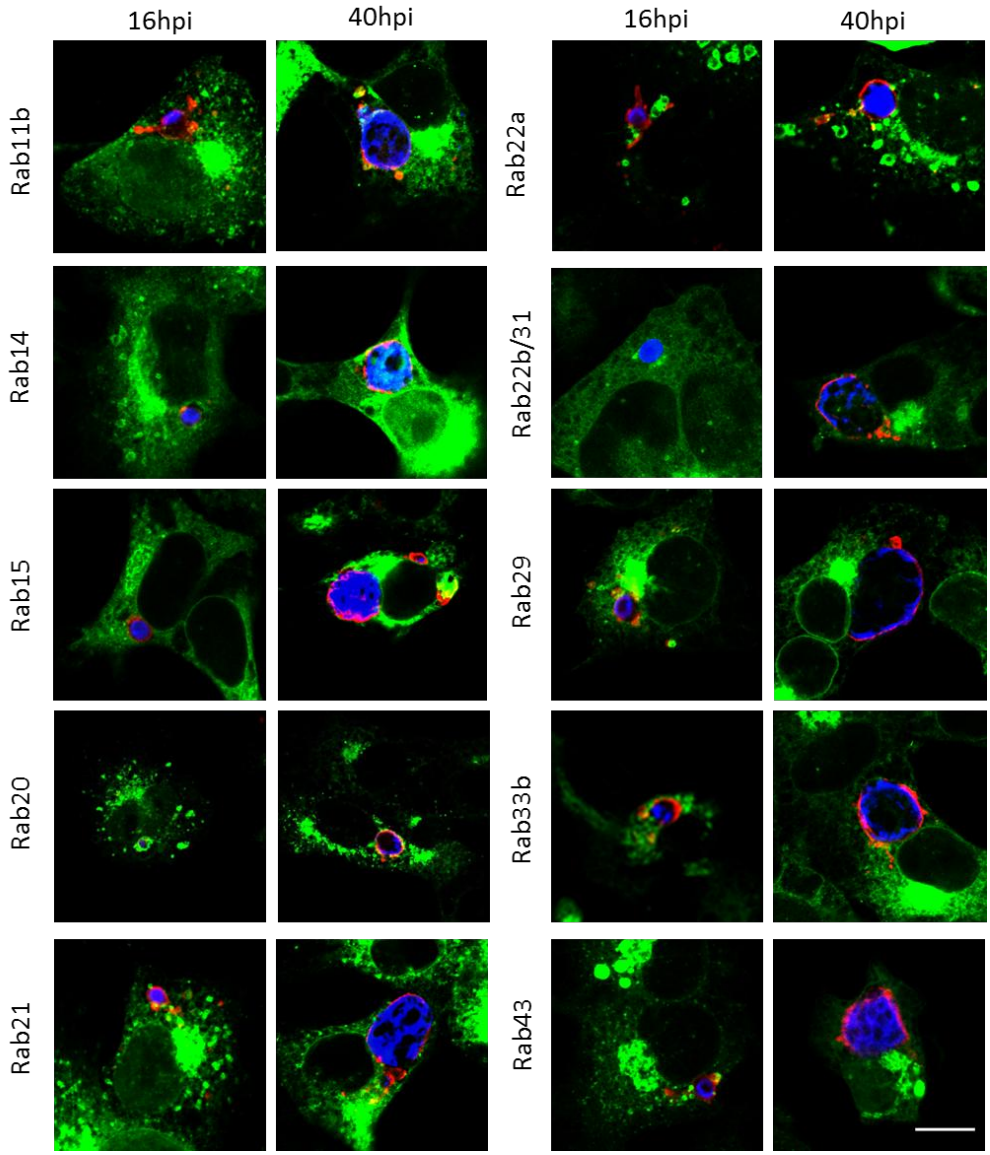


Figure 3.3– GFP-Rab localization during P.berghei liver infection. Hepa1-6 cells were transduced with GFP-Rab (green) the day before infection with *P.berghei* parasites. Infection was stopped at 16 and 40 hours post infection, and parasites were stained with anti-UIS4(red) and 2E6 (blue). Scale bar: 10µm.

Rab9 was seen accumulating around the parasite vacuole, as expected, similar to Rab7a (see **Figure 2.9**) since these Rabs are involved in late endosome/lysosome trafficking. Interestingly, a number of other Rab were also seen to interact with the parasite at some stage during liver infection. These

were Rab1a, Rab1b, Rab6a, Rab8a, Rab20 and Rab43 (discussed later). As expected, Rabs traditionally associated with early endosomes and recycling endsomes, such as Rab22 (Magadán et al., 2006)(Zhu et al., 2009) and Rab5 (shown previously in **Figure 2.8**) were generally not seen associating with parasites.

Curiously, a number of Rabs found accumulating around parasites, such as Rab 6, Rab8 and Rab43 are involved in aspects of Golgi trafficking and maintenance, emphasizing the fact that parasites may interact with this host organelle. Unfortunately, when Rab6 and Rab8 were depleted in Hepa1-6 using siRNA, no effect on parasite infection rate was seen (see **Supplementary Table 3**), although only one time point was tested and parasite size was not measured. Rab43 depletion was not performed. Thus, the role of these proteins during Plasmodium liver infection remains to be elucidated.

It is important to note that, using a protein overexpression system, instead of antibodies against endogenous protein, may create artifacts in the membrane targeting of Rab proteins, especially when the equilibrium between cytosolic and membrane bound Rab is altered by having an abnormal amount of protein in a cell. Thus the colocalization results above were taken with great care since the distribution of each overexpressed Rab could be different from the endogenous protein. However, Rab1a was a functional target within the siRNA screen as well as showing colocalization with the parasite vacuole in the overexpression studies making it an interesting target for further analyses in *Plasmodium* infection.

3.3 siRNA screen hit – Rab1a knock-down increases *Plasmodium* liver infection

Only one significant hit was obtained in this study and unexpectedly, this was Rab1a, a protein known to be involved mostly in ER-to-Golgi trafficking. In mammals, Rab1a is closely related to the Rab1b isoform (nearly

80% sequence similarity) and is highly expressed in most animal tissues (Su et al., 2004). Under normal conditions, Rab1a localizes to membranes of the *cis* Golgi-intermedial compartment and is responsible for the control of vesicle traffic from the endoplasmic reticulum to the Golgi apparatus (Allan et al., 2000) as well as intra-Golgi transport (Moyer et al., 2001). Rab1 has also been implicated in maintenance of Golgi integrity and functionality (Haas et al., 2007)(Bannykh et al., 2005) as well as being involved in secretory pathways in mammalian cells (Saraste et al., 1995).

The Golgi apparatus is a compact organelle that plays a central role in anterograde transport of newly synthesized proteins from the ER to the plasma membrane or to other intracellular organelles (Sannerud et al., 2003b). Recently, new roles for the Golgi apparatus, such as receiving incoming traffic from endocytic and recycling pathways, cytoskeletal dynamics, organelle biogenesis, receptor signaling and apoptosis have also been shown (Derby and Gleeson, 2007), thereby suggesting a highly dynamic and active organelle.

More recent studies have implicated Rab1a during autophagy as Rab1a was seen to localize with autophagosomes and its activity was required for early autophagosome formation, which is in agreement with the emerging theory that some part of autophagosome formation involves the ER (Mijaljica et al., 2006)(Hayashi-Nishino et al., 2009)(Huang et al., 2011). Rab1a overexpression promotes autophagosome formation as previously described in yeast, where the autophagic process is induced in cells with an effective block in the hydrolysis of Ypt1p (Rab1 homolog)-bound GTP. (De Antoni et al., 2002). The capacity to form autophagosomes was significantly decreased in mammalian cells with decreased levels of Rab1a.

Interestingly, there are intracellular pathogens that interact with host Rab1a in order to establish their intracellular niche. This is the case for *Legionella pneumophila*, where a large body of evidence has shown that this bacterium subverts host Rab1a function by producing bacterial proteins that directly interact with this host protein (Kagan 2004).

All this data suggests that apart from its established role in ER-to-Golgi trafficking role, Rab1a also plays a crucial role in mammalian autophagy. Thus we proceeded to analyze the role of Rab1a during *Plasmodium* liver infection and also which of these two pathways was most significant in affecting *Plasmodium berghei* liver infection.

3.3.1 Rab1 effects *Plasmodium berghei* liver infection

In order to confirm the results obtained in the siRNA screen, Hepa1-6 cells were transfected with an siRNA pool targeted against murine Rab1a and infected with *P.berghei* sporozoites. Infection was stopped by fixing cells at different times post infection (16 and 40hpi). The total number of parasites per well and the size of EEFs was measured and compared to control wells. The level of mRNA knock-down at the time of infection was also quantified (**Figure 3.4**).

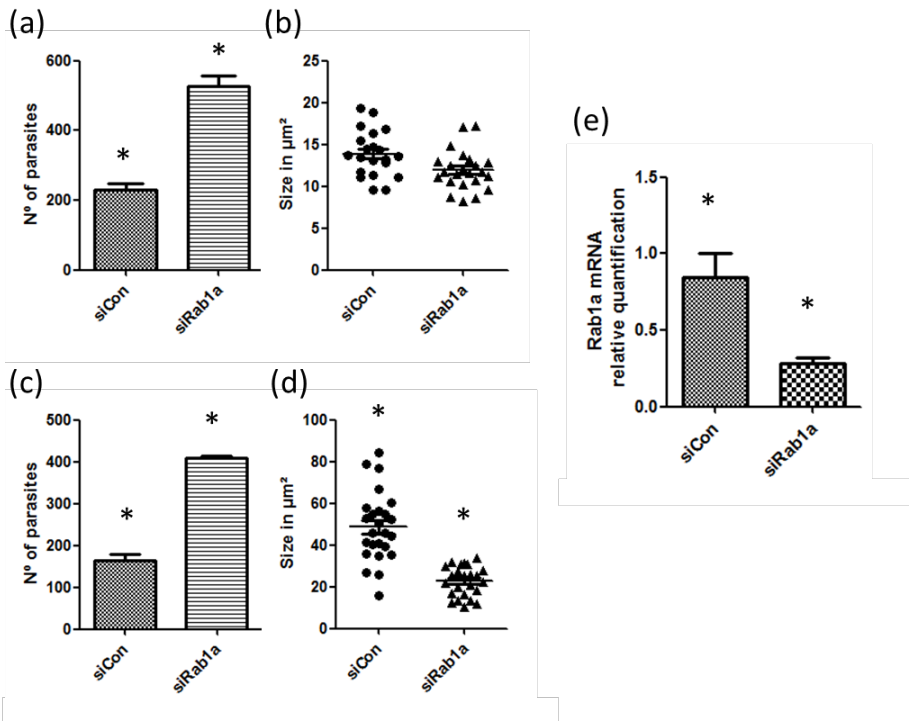


Figure 3.4 – Rab1a downregulation affects both Plasmodium infection rate and size. Hepa1-6 cells were treated with siRNA targeting Rab1a and infected with *P.berghei* sporozoites. Cells were fixed at either (a)/(b) 16 hour post infection or (c)/(d) 40 hours post infection. (a)/(c) mean total number of parasites per well and (b)/(d) EEF size was measured. (e) Rab1a knock-down, at the time of infection, was measured by qRT-PCR. * P-value: 0.0001.

As expected, **Figure 3.4** shows that decreasing the amount of Rab1a cells causes *P.berghei* infection rate to almost double, even when measured at 16 hours post infection. This would suggest that Rab1a is having an effect on parasite survival during the initial stages of infection. Parasite size, at this point, is not affected. Surprisingly, the size of parasites was significantly smaller at 40 hours, suggesting that Rab1 may have a role in parasite growth during the late stage of liver *Plasmodium* infection.

To rule out any involvement of Rab1a during parasite migration, which could account for the differences in infection rate, parasite migration was assayed, using a Calcein Migration assay (see Materials & Methods). Briefly, cells were incubated with Calcein Green AM which is retained in the cell

cytosol. Cells were washed, infected with sporozoites and parasites were allowed to migrate and invade cells for 1 hour. During migration, the plasma membrane of cells is ruptured and Calcein Green is released, which is subsequently measured in the growth medium using a spectrophotometer. As shown in **Figure 3.5**, no difference in migration was seen between control and Rab1 knock-down cells, even when both Rab isoforms were used, indicating that Rab1a is not having an effect on the migration of *P.berghei* parasites but possibly during the post invasion stage of parasite liver development, even if within the first hours of invasion.

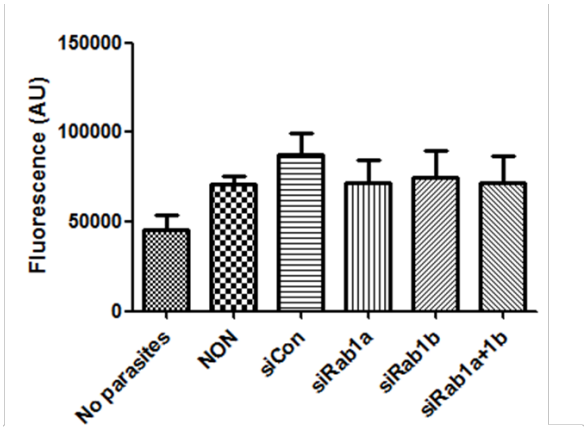


Figure 3.5 – Rab1a does not affect Plasmodium migration in liver cell. siRNA control and treated Hepa1-6 cells were incubated with Calcein Green AM which is retained in the cell cytosol. Cells were washed, infected with sporozoites and parasites were allowed to migrate and invade cells for 1 hour. During migration, the plasma membrane of cells is ruptured and Calcein Green is released, which is subsequently measured in the growth medium using a spectrophotometer.

In order to further characterize the effects of Rab1a during *P.bergei* infection, Hepa1-6 cells were transduced with either GFP alone or GFP-Rab1a virus constructs, effectively increasing the amount of Rab1a by an average of four times when measured using qRT-PCR (see **Figure 3.6**). The effect of overexpressing Rab1a on parasite infection was analyzed by measuring the total number and sizes of EEFs at two different times post infection.

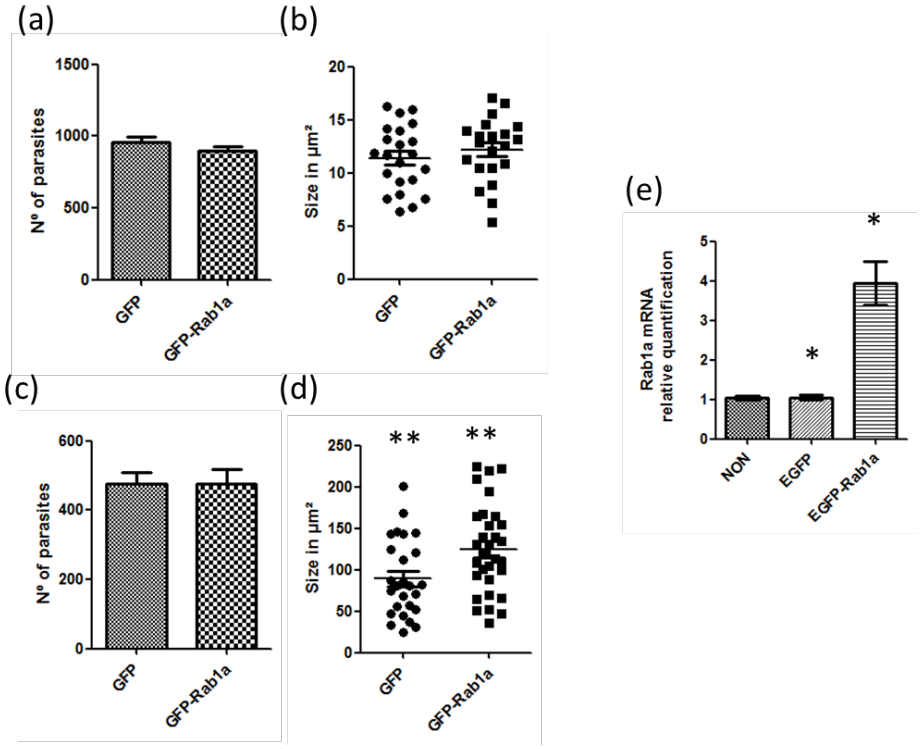


Figure 3.6 – ***Rab1a overexpression effects parasite size***. Hepa1-6 cells were transduced with either GFP alone or GFP-Rab1a 24 hours prior to infection with *P.berghei* sporozoites. Cells were fixed at either (a)/(b) 16 hour post infection or (c)/(d) 40 hours post infection. (a)/(c) mean total number of parasites per well and (b)/(d) EEF size was measured. (e) Rab1a overexpression, at the time of infection, was measured by qRT-PCR. * P-value: 0.0001; ** P-value: 0.0108.

Overexpressing Rab1a unexpectedly did not alter parasite infection rate during the initial stages of infection but this could be due to the low expression of Rab1a in these cells, since the expression level of Rab1 was controlled to be moderate, to avoid death of cells expressing too much GFP. Nevertheless, parasite size at 40 hours post infection was only moderately but significantly increased, once again suggesting a role of this protein in parasite growth during the late stage of infection (P-value 0.0108).

The localization of Rab1a in infected cells was analyzed by transducing cells with GFP-Rab1a, infecting with *P.berghei* sporozoites and stopping infection at various times. As expected, GFP-Rab1a showed the expected ER-

Golgi staining pattern, concentrating in the perinuclear area. More than 90% of parasites were found very close to this organelle (within 3µm), suggesting again a strong interaction between this organelle and *Plasmodium* parasites.

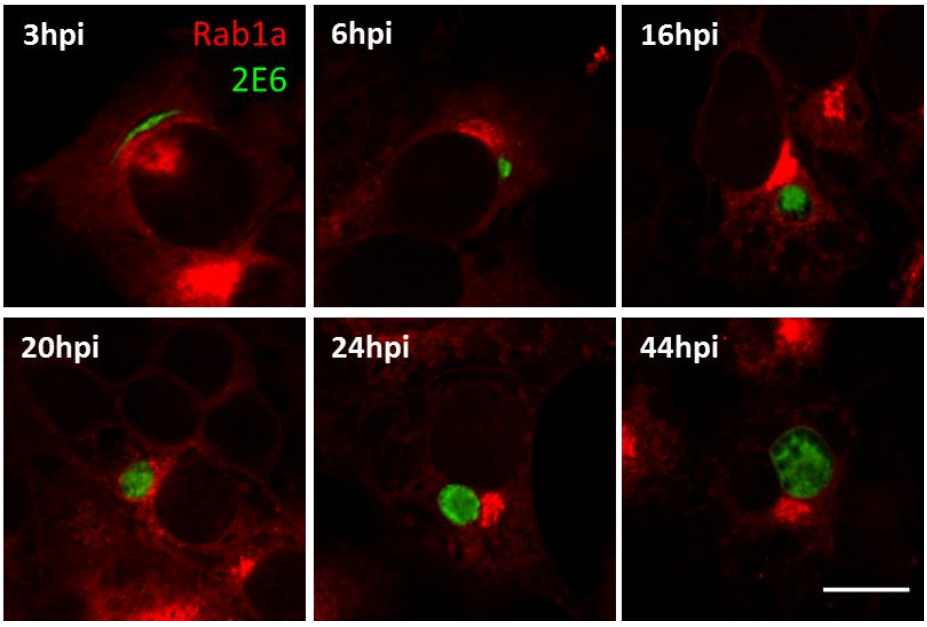


Figure 3.7 – P.berghei parasites localize closely with host Rab1a. Hepa1-6 cells were transduced with GFP-Rab1a (red) 24 hours prior to infection with *P.berghei* sporozoites. Cells were fixed at various times post infection, as indicated, and stained with 2E6 antibody (green). Scale bar: 10µm.

3.3.2 Rab1 affects Plasmodium infection via the autophagic pathway

As mentioned before, Rab1 has been implicated with the autophagic pathway, more specifically, in initial autophagosome formation, as well as its role in ER-to-Golgi traffic. The role of Rab1 in autophagy seems to be independent from its role in ER-to-Golgi transport (Huang et al., 2011). Thus we proceeded to investigate whether Rab1a was affecting parasite liver infection via the ER-to-Golgi route or by affecting the autophagic pathway.

Sar1 is a small GTPase that has been shown to be involved in the formation of COPII-coated transport vesicles that bud from the ER (Takai et

al., 2001). Overexpressing the dominant-negative mutant (Sar1-T39N) has been shown to disrupt the secretory pathway by disassembling the Golgi apparatus and inhibiting vesicle budding from the ER (Yoshimura et al., 2004).

Brefeldin A is a widely used drug that causes disassembly of the Golgi apparatus (Dinter and Berger, 1998), causing it to mix with the ER, and causes inhibition of protein secretion, as proteins are retained in the ER (Klausner et al., 1992). Although it is not known if this drug affects *Plasmodium* parasites in the liver, siRNA targeting Sar1 and Brefeldin A treatment to cells was used to determine whether the Rab1a effect on parasite infection was through the ER-to-Golgi transport pathway.

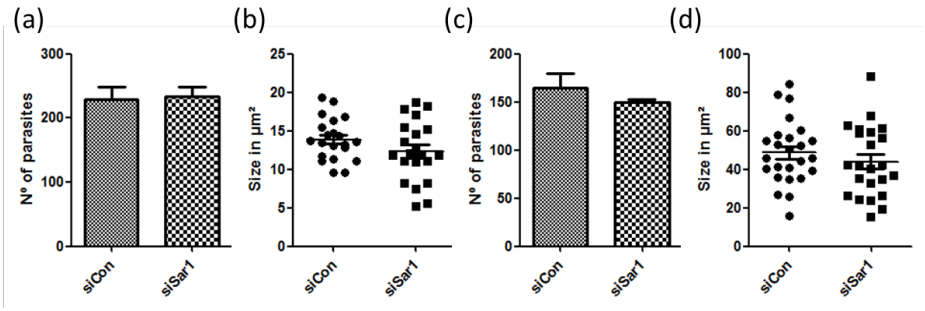


Figure 3.8 – Sar1 downregulation does not affect Plasmodium liver infection. Hepa1-6 cells were treated with siRNA targeting Sar1 and infected with *P.berghei* sporozoites. Cells were fixed at either (a)/(b) 16 hour post infection or (c)/(d) 40 hours post infection. In (a)/(c) mean total number of parasites per well and (b)/(d) EEF size was measured.

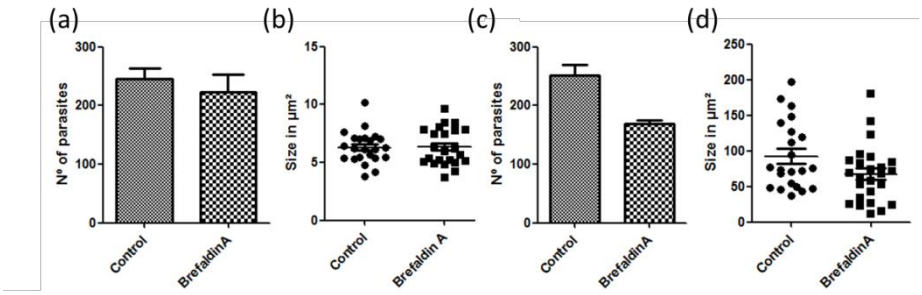


Figure 3.9 – Brefeldin A treatment has no effect on Plasmodium liver infection. Hepa1-6 cells were infected with *P.berghei* parasites and treated one hour post infection with Brefeldin A (75ng/ml) for the initial 16 hours of infection. Cells were fixed and stained with 2E6 and DAPI and total EEF number (a) and size (b) were measured. (c)/(d) Cells were infected with *P.berghei* parasites and treated with Brefeldin A (75ng/ml) from 16 to 40 hours post infection. Cells were fixed at 40 hours post infection and total number of EEFs (c) and EEF size (d) was measured.

From **Figure 3.8** and **Figure 3.9**, it is possible to conclude that inhibiting ER-to-Golgi trafficking by down regulating Sar1 or Brefeldin A treatment, had no effect on either parasite infection rate (measured by counting the total number of EEFs per well) and mean EEF size. These results raise the possibility that it is the role of Rab1a in autophagy that is important during Plasmodium infection.

Autophagy is a process by which host cytoplasmic contents are degraded and recycled (Kuma and Mizushima, 2010). This process is highly regulated and initiates with the formation of an autophagosome which ultimately fuses with a lysosome and degrades the internalized material. Autophagy also has an established role in cellular antibacterial destruction and elimination (Levine and Kroemer, 2008). There are various methods of inducing or down regulating autophagy in cells (Mizushima et al., 2010). One of the methods often used is to down-regulate proteins essential for autophagosomes formation, such as the vacuolar sorting protein 34 (VPS34).

When Hepa1-6 cells were down-regulated for VPS34, inhibiting autophagosome formation, more parasites were able to survive during the first 16 hours compared to control cells (**Figure 3.10**), just like what happened when cells were down-regulated for Rab1a (**Figure 3.4**). Also similar to the

results with Rab1a, when VPS34 was down-regulated, EEFs were smaller at 40 hours post infection, suggesting a role for autophagy during the late stages of parasite growth.

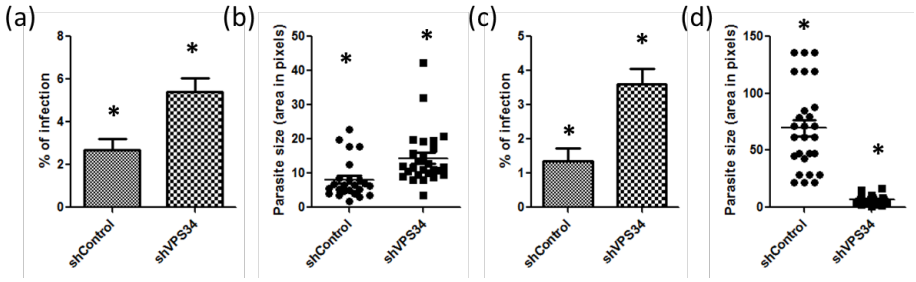


Figure 3.10 – Inhibition of autophagosome formation effects Plasmodium infection and development. Hepa1-6 cells were down-regulated for VSP34 and infected with *P.berghei* parasites. (a)/(c) Total number of EEFs at 16 and 40 hours post infection respectively was quantified and (b)/(d) EEF size was measured at 16 and 40 hours post infection respectively. Preliminary experiment performed by Laura Santos.

These preliminary results suggest that Rab1a is primarily effecting *Plasmodium* infection through its role in autophagy. Although further studies are required, these results suggest a novel role for autophagy during *Plasmodium* liver infection, which is being further investigated in the lab.

In order to characterize the localization of Rab1a during *P.berghei* infection, Hepa1-6 cells were transduced with GFP-Rab1a, infected, fixed and stained with microtubule-associated protein 1 light chain 3 (LC3) antibody, a widely used marker of autophagic vesicles (**Figure 3.11**). Strikingly, LC3 positive vesicles aggregated significantly around the parasite during the entire liver infection. A small proportion of Rab1a vesicles were LC3 positive, suggesting that Rab1a may be involved in the initial formation of autophagic vesicles. Taken together, these experiments strongly suggest that autophagy is playing a role during *Plasmodium* liver infection.

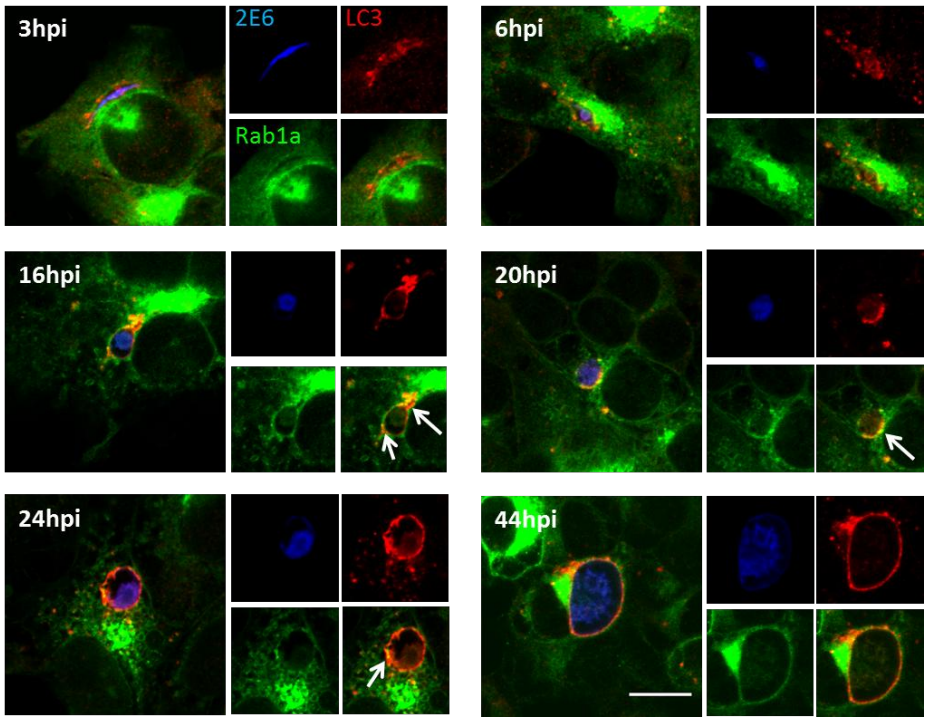


Figure 3.11 – Rab1a colocalizes with autophagic vesicles which aggregate around the parasite. Hepa1-6 cells were transduced with GFP-Rab1a (green) 24 hours prior to infection with *P.berghei* sporozoites. Cells were fixed at various times post infection and stained with 2E6 (blue) to stain the parasite cytoplasm and anti-LC3 antibody (red). It is possible to notice some (arrows), although very few, Rab1a/LC3 positive vesicles, shown in yellow. Scale bar: 10µm.

It is well established that not all parasites that invade cells (*in vitro*) are able to develop fully and form merozoites, although the reason and mechanism behind this effect has been largely uncharacterized. **Figure 3.12** shows a parasite survival curve, where various wells with Hep1-6 cells were infected with the same number of parasites but stopped at various times post infection and the total number of parasites per well was calculated for various time points. It is clear to see that there is a very rapid decrease in parasite numbers during the initial stages of infection, where parasites seem to be especially prone to being eliminated by the host cell. This is also when there is little parasite growth (see **Figure 2.4**) and presumably when parasites are adapting and creating their new intracellular niche.

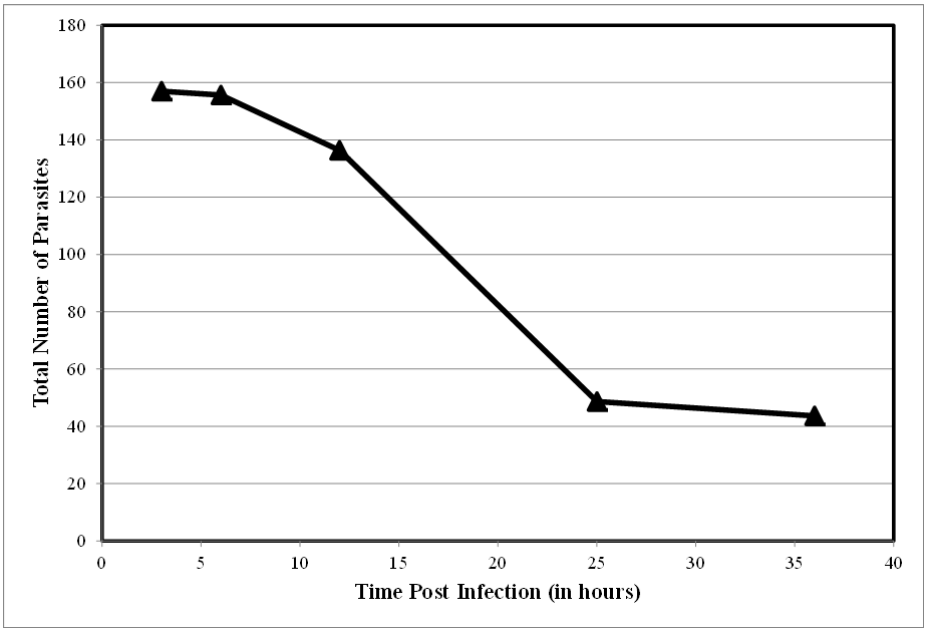


Figure 3.12 – Plasmodium berghei survival kinetics in Hepa1-6 cells. Hepa1-6 cells were infected with the same number of *P.berghei* parasites and samples were fixed at various times post infection (x-axis). Total number of parasites per well was quantified (y-axis).

Later during infection (after 25-30 hours), parasite numbers seem to stabilize, suggesting that parasites become less sensitive to host cell killing at this stage. This suggests that parasites that do reach this stage of liver infection have adapted well to their new niche and have reached an equilibrium between the host cellular defense mechanisms and its own requirement to interact with the host cell in order to acquire nutrients. This is when most parasite growth occurs and when a bigger interchange with the host cell is required.

Taken together, these results suggest that *P.berghei* liver development may be divided into three stages: (1) an initial stage when parasites are establishing their niche and the initial morphological transformations occur, (2) a second phase, between 12 and 20 hours post infection, when parasites are very susceptible to host cell killing, possibly by the host cell lysosomal and/or autophagy pathway, and (3) a later growth phase, when parasites are no longer sensitive to host cell killing but instead require host cells for nutrient acquisition.

Although the role of Rab1a during *P.berghei* liver infection seems to be related with its role in autophagy, the role of the late endosomes/ lysosomes shown aggregating around *Plasmodium* parasites in the previous Chapter is still unknown. The next chapter will attempt to characterize this host-parasite interaction in more detail.

3.4 Discussion

siRNA technology is now a widely used method to study host-pathogen. With the development of more efficient and automated high-throughput technology, entire genomes have now been tested in the context of pathogen infections. Nevertheless, at the time when this study was initiated, these high-throughput methods were still being developed and thus we opted to develop a more specific and smaller assay, to study a specific family of protein, the Rab GTPases, and their role during *Plasmodium* liver infection.

Very surprisingly, only one protein, Rab1a, seemed to have an effect on *Plasmodium berghei* liver infection rate at 24 hours post infection. It should be noted that the present data does not fully exclude the involvement of other Rabs amongst those tested and that only time point was measured. Thus the role of other Rab protein during the late stages of infection remains to be tested.

Overexpression studies, using GFP-Rab virus constructs, were performed and the location of 20 Rabs in relation to *Plasmodium* parasites was tested. Interestingly, a few unexpected Rabs seemed to interact with parasites in some way. As expected, Rab9 was seen accumulating around the parasite vacuole, similar to Rab7 since these Rabs are involved in late endosome/lysosome trafficking.

Interestingly, a number of other Rab were also seen to interact with the parasite at some stage during liver infection. These were Rab1a, Rab1b, Rab6a, Rab8a, Rab20 and Rab43.

Rab20 was found to aggregate around parasites at 16 hours post infection. Rab20 has been reported to localize to the ER (Das Sarma et al., 2008) but was also shown to colocalize with vacuolar-type ATPases (Curtis and Gluck, 2005). More recently, it was shown to be involved in both phagosome acidification and Cathpesin D recruitment to the *M.tuberculosis* phagosome (Seto et al., 2011). These findings suggest a role of Rab20 in phagosome maturation. Although not a hit in the siRNA screen, these findings together with its localization around *Plasmodium* parasites in this study makes Rab20 a candidate meriting further study.

Rab6a, Rab8a and Rab43 are Rabs involved in aspects of Golgi trafficking and maintenance, emphasizing the fact that parasites may interact with this host organelle. More recently, Rab43 was also found to regulate the recruitment of Cathepsin D to the phagosomes of *M.tuberculosis* and *S.aureus* (Seto et al., 2011) suggesting a new role for Rab43 in phagosome maturation, perhaps utilizing a direct pathway from the Golgi. Unfortunately, when Rab6a and Rab8a were depleted in Hepa1-6 using siRNA, no effect on *P.berghei* parasite infection rate was seen (see **Supplementary Table 3**), which is congruent with the results obtained with Brefeldin A, although only one time point was tested and parasite size was not measured. Rab43 depletion was not performed. Thus, the role of these proteins during *Plasmodium* liver infection remains to be elucidated.

Since Rab1a was a functional target within the siRNA screen as well as showing colocalization with the parasite vacuole in the overexpression studies, we believed it was an interesting target for further analyses during Plasmodium infection.

Rab1a is a protein known to be involved mostly in ER-to-Golgi trafficking. Yet, more recently, Rab1 has been implicated to be important during the process of autophagy, more specifically during autophagosome formation (Huang et al., 2011). Thus the role of Rab1a during *Plasmodium* liver infection was investigated to try to dissect which was most significant pathway affecting *Plasmodium berghei* liver infection.

Since the siRNA was only performed by analyzing parasites at 24 hours post infection, we repeated the experiment using other time points and measuring not only parasite infection rate by also parasite size. As expected, total number of parasites was doubled in Rab1a depleted treated cells compared to control, just as in the screen. Surprisingly, Rab1a also had an effect in parasite growth during the later stages of infection, since EEFs in Rab1a depleted cells were almost half the size compared to control.

When Rab1a was overexpressed in cells, parasite infection rate was surprisingly not effected, although parasite size at 40 hours post infection was slightly increased. In order to maintain cell viability and integrity, Rab1a expression levels were controlled to avoid having cells overexpressing too much GFP protein. This could explain why parasite infection was not effected in these experiments even though EEF size was increased. Colocalization experiments showed that around 90% of parasites, at all time points tested, were found very close to this protein, suggesting a strong interaction between *Plasmodium* parasites and host Rab1a.

In order to dissect whether Rab1a was effecting *Plasmodium* infection via de ER-to-Golgi route or via the autophagic pathway, Brefeldin A and treating cells with siRNA targeting Sar1, both treatments described to block ER-to-Golgi trafficking, were used. None of these treatments had an effect in parasite infection rate and size, suggesting that it is the role of Rab1a during autophagy that is affecting *Plasmodium* infection.

Interestingly, preliminary evidence in our lab also suggested a role of autophagy during *Plasmodium berghei* liver infection. As shown in **Figure 3.10**, when autophagy was inhibited by knocking-down vacuolar sorting protein 34 (VPS34), a protein know to be involved in autophagosome formation (Funderburk et al., 2010) had the same effect on parasites as when in cells treated with siRNA targeting Rab1a. When infected cells were transduced with GFP-Rab1a and stained with microtubule-associated protein 1 light chain 3 (LC3) antibody, a widely used marker of autophagic vesicles, some of the vesicles seen surrounding parasites were positive for both markers.

Taken together, these experiments strongly suggest that autophagy is playing a role during *Plasmodium* liver infection which is being further investigated in the lab. Nevertheless, at this point, and from the results so far, it seems that autophagy is playing a dual role during *Plasmodium* liver infection. When autophagy is inhibited, more parasites are able to survive the initial hours of infection, where parasites are more sensitive to dying, suggesting a possible role of autophagy in this early stage parasite elimination. During late infection, when parasite are replicating and growing at an astonishing rate, inhibition of autophagy decreases parasite size, suggesting a role in parasite development, possibly as a source of nutrients and lipids.

Together with pervious data which showed that late endosomes/lysosomes were seen aggregating around parasites, the identity and role of these vesicles will be further characterized.

4. Dissecting *Plasmodium*-lysosome interactions

All the work presented in this chapter was performed by Mafalda Lopes da Silva with the help of Carolina Matos.

Summary

Previous data has showed that various membrane markers, such as Rab7a, CD63 and LAMP1, all present on late endosomes, multivesicular bodies (MVBs) and lysosomes, as well as the autophagic marker, LC3, found on amphisomes and autophagosomes, aggregate around *Plasmodium* parasites throughout the entire liver infection. Further studies indicated that these vesicles are also acidic but surprisingly, Cathepsin D negative.

In order to characterize the nature and role of these vesicles during *Plasmodium* liver infection, infected cells were treated with ammonium chloride, a treatment that dissipates the pH gradient within cells, inhibiting the trafficking and maturation of late endosomes, MVBs and lysosomes. This treatment had no effect on parasite infection rate but severely impaired parasite growth, especially during the late stages of infection where mean exo-erythrocytic forms (EEF) size decreased threefold. A similar result was obtained with Concanamycin A, a drug that acts as a vATPase inhibitor.

Taken together, these observations suggest that parasites are surrounded by MVBs and/or amphisomes that contain the membrane markers Rab7a/CD63/LAMP1/LC3 but are not mature lysosomes, since they are Cathepsin D negative. Since disturbing the trafficking of these vesicles effects parasite development, taken together, these results point toward a new role for these host vesicles during *Plasmodium* parasite late liver infection, more specifically, as a possible source of nutrients for parasite growth. This correlates well with the results obtained with Rab1a, where overexpression of Rab1a, effectively up-regulating autophagosome formation, resulted in larger EEFs, possibly as the abundance of amphisomes increased in these cells. The opposite occurred when Rab1a was downregulated. Transmission electron microscopy images also corroborate the concept that these vesicles could be fusing with the PVM, pointing toward a novel role for amphisomes as a source of nutrients during *Plasmodium* liver infection.

Materials & Methods

Cell lines, Parasite strains, microscopy sample preparation, virus production siRNA transfection and qRT-PCR, and electron microscopy preparation.

All performed as described in Chapter 2.

HUH7 cells were generously provided by M.M.Mota (IMM, Lisbon) and cultured in RPMI medium (Gibco/Invitrogen) with 10% FCS (Gibco/Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco/Invitrogen), 1% non-essential amino acids (Gibco/Invitrogen) and 1% HEPES (Gibco/Invitrogen). Cells were maintained in a humidified cabinet at 37 ^{0}C and 10% CO_2.

Antibodies

Apart from the antibodies already described in Chapter 2&3, rabbit polyclonal anti-Cathepsin D antibody was utilized (Upstate; 1:100).

Live cell imaging

Hepa1-6 cells ($4x10^4$) were seeded on glass bottom culture dishes (MatTek Corporation) and infected with $5x10^4$ GFP-*P.berghei* sporozoites. Samples were visualized using an inverted Leica SP5 confocal microscope with a resonance scanner and fitted with a temperature and CO_2 control chamber.

To visualize lysosomes, LysoTracker®Red (Invitrogen) was diluted 1:20,000 in growth medium and added to cells 5 minutes prior to image acquisition. To visualize acidic vesicles using pHrodo™dextran (Molecular Probes/Invitrogen), cells were incubated with pHrodo™dextran (70 µg/ml) for 12-16 hours, then washed in growth medium and incubated for a further 2-3 hours in growth medium, to accumulate the dye in the lysosomal pathway. Samples were analyzed using an inverted SP5 confocal microscope with a

resonance scanner and fitted with a temperature and CO_2 control chamber. Acquisition settings were not changed between different samples.

Drug concentrations

Ammonium Chloride (NH_4Cl) (Sigma) was incubated with cells at a final concentration of 25mM in growth medium. Concanamycin A (Sigma) was used at 0.1nM in growth medium.

At this point, a number of membrane markers have been shown to be closely associated with the PVM during the entire liver stage infection. These are Rab7a, CD63, LAMP1 (all present on late endosomes/ multivesicular bodies (MVBs) and lysosomes) as well as the autophagic marker, LC3, which may be found on amphisomes and autophagosomes. Thus, any of these types of vesicles could be interacting with parasites. This Chapter further dissects which of these vesicles could be interacting with parasites and what is their role during *Plasmodium* liver stage development.

A key feature of the lysosomal pathway is that vesicles get progressively acidic (see Chapter 1.7 for a more detailed description of this process). Thus we proceeded to investigate whether the vesicles that surround *Plasmodium* parasites in liver cells contained an acidic lumen.

4.1 Plasmodium is surrounded by acidic vesicles but avoids acidification

One of the most important factors in phagosome maturation is fusion of the phagosome membrane with acidic vesicles, releasing the highly acidic and degradative contents, ultimately leading to phagosome content degradation. For this reason, various intracellular pathogens have evolved strategies to avoid the fusion and acidification with host lysosomes (see Chapter 1.8). This was tested in the context of a malaria infection in liver cells.

One way in which fusion is often tested is by measuring the pH of the phagosome lumen, since fusion with lysosomes acidifies this vacuole. To do this, live imaging microscopy techniques were used since dyes sensitive to the pH of intracellular vesicles were developed for live cell imaging.

The most widely used fluorescence dye to stain lysosomes in living cells is LysoTracker®Red (Invitrogen). This dye is freely permeable to cell membranes and selectively accumulates in cellular compartments with a low internal pH. Due to its ease of use it has been extensively used as a marker to

visualize the dynamics of lysosome movement in live cells (Chazotte, 2011). Thus LysoTracker®Red was used in the context of parasite infection, in order to study the dynamics of host lysosomes around *P.berghei* parasites.

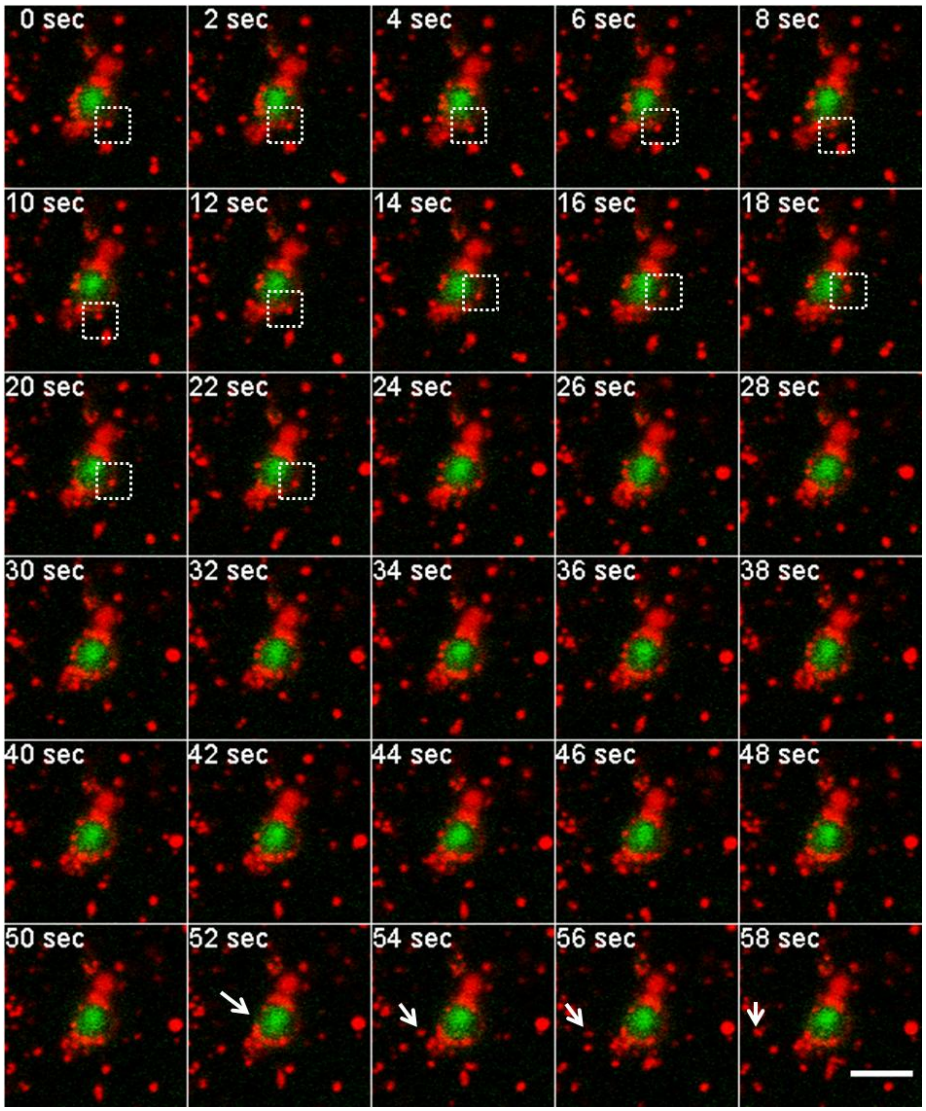


Figure 4.1 – LysoTracker®Red vesicles aggregate but do not fuse with the P.berghei PVM. Hepa1-6 cells where seeded on glass bottom culture dishes (MatTek Corporation) and infected with GFP-*P.berghei* sporozoites. 5 minutes prior to visualization, cells were incubated with LysoTracker®Red (1:20 000) in grwoth medium. Cells were visualized using an inverted SP5 Leica confocal system equipped with a resonance scanner. Time lapse images of LysoTracker®Red (red) and a 16 hour GFP-*P.berghei* parasites (green) are shown. A vesicle that moves towards the parasite and stabilizes on the PVM (dashed square) and another that moves away from the PVM (arrow) are shown. Scale bar: 5µm.

Time-lapse microscopy allows the visualization of specific vesicles location and their dynamics and movement, a dimension that is lost in fixed cells. As expected, LysoTracker®Red positive vesicles were seen to cluster around the parasite but more interestingly, some vesicles are seen to become stabilized on the PVM and stop movement altogether. Some vesicles were even seen to come into close contact with the PVM and then leave again (**Figure 4.1**). Interestingly, the area occupied by the parasite was always negative for LysoTracker®Red, indicating that the parasite vacuole itself never became acidified.

At the same time two different visual controls were established; a positive control, using latex beads, which have been described to undergo normal phagocytosis in hepatocytes and thus were used as an example of lysosomal fusion. As a negative control, GFP-*Toxoplasma gondii* parasites were used, as it has been shown that they do not aggregate nor fuse with host lysosomes (Jones and Hirsch, 1972)(Sibley et al., 1985).

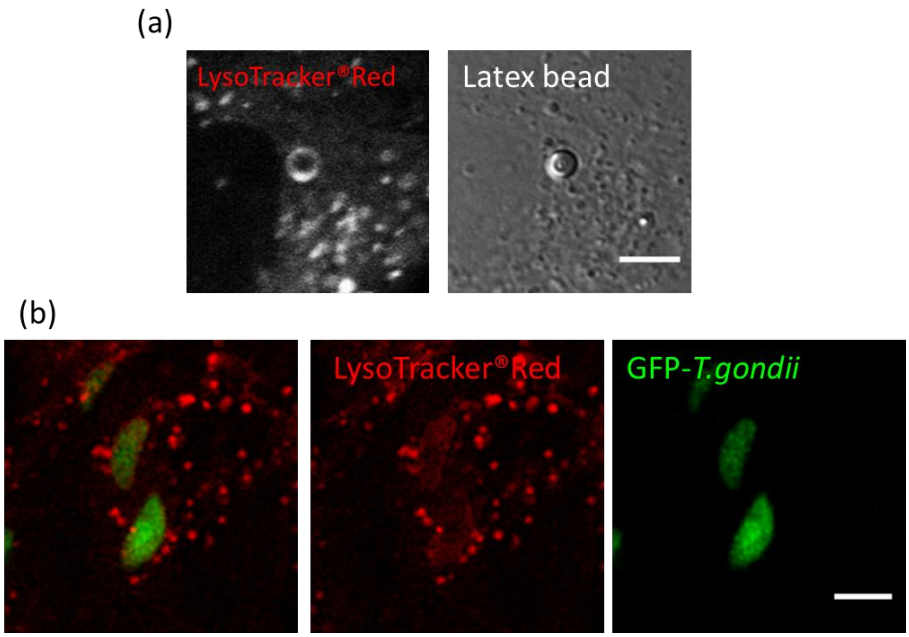


Figure 4.2 – LysoTracker®Red vesicles fuse with latex beads but not with T.gondii parasites. Hepa1-6 cells where seeded on glass bottom culture dishes (MatTek Corporation) and infected with either (a) latex beads, or (b) GFP-*Toxoplasma gondii*. Infection was left to proceed for 6 hours. 5 minutes prior to visualization, cells were incubated with LysoTracker®Red (1:20 000) in growth medium (red). Cells were visualized using an inverted SP5 Leica confocal system equipped with a resonance scanner. Scale bar: 10µm.

Figure 4.2 shows very clearly that the staining pattern between the two conditions, fusion and no fusion is very easily distinguished. In the case of fusion of lysosomes with the latex beads, the vacuole retains a continuous rim of red fluorescence, indicating that the internal side of the vacuole membrane becomes LysoTracker®Red positive. In the case of *Toxoplasma gondii* parasites, as expected, no specific clustering or fusion is seen around the parasite vacuole.

In order to confirm these results, another pH sensitive dye was used. pHrodo™dextran (Invitrogen) enters the cell through endocytosis and is a pH-sensitive dye that is non-fluorescent in neutral pH and fluoresces bright red as the pH decreases. Cells that were incubated with this dye for 12-16 hours followed by washing and further incubation in growth medium for a 2-3 hour period (chase) accumulated pHrodo™dextran in the lysosomal pathway. This

dye does not retain well in cells after fixation, but when cells were incubated using this protocol another dextran-Alexa647 of the same molecular weight, together with LysoTracker®Red, all the stained vesicles were positive for both dyes, indicating that this protocol ensures that this dye is concentrating in late endosome/ lysosome pathway (**Figure 4.3**).

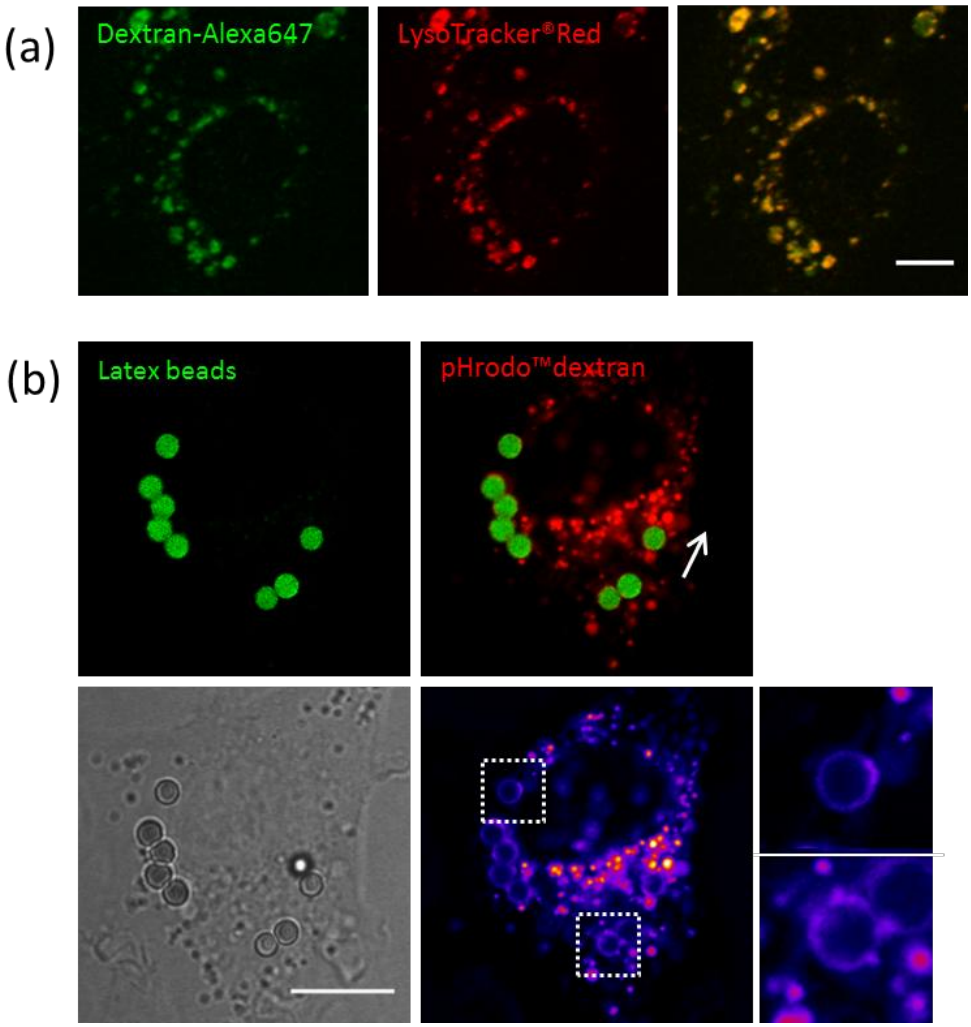


Figure 4.3 – pHrodo™dextran stains acidic lysosomes. (a) Hepa1-6 cells on glass bottom culture dishes (MatTek Corporation) were incubated with Dextran-Alexa647 (green) according to the protocol described. 5 minutes prior to visualization, cells were incubated with LysoTracker®Red (1:20 000) in growth medium (red). Scale bar: 20µm. (b) Hepa1-6 cells were incubated with latex beads (green and bright field image) prior to incubation with pHrodo™dextran (red and pseudocoloured image where less intense vesicles (more neutral) are purple and more intense vesicles (more acidic) are shown in pink and yellow. Scale bar: 10µm.

Hepa1-6 cells were infected and incubated with pHrodo™dextran according to the protocol described. Since this dye´s fluorescence intensity signal is directly proportional to the acidity of the vesicles, it is possible to visualize and compare the acidity of lysosomes in cells.

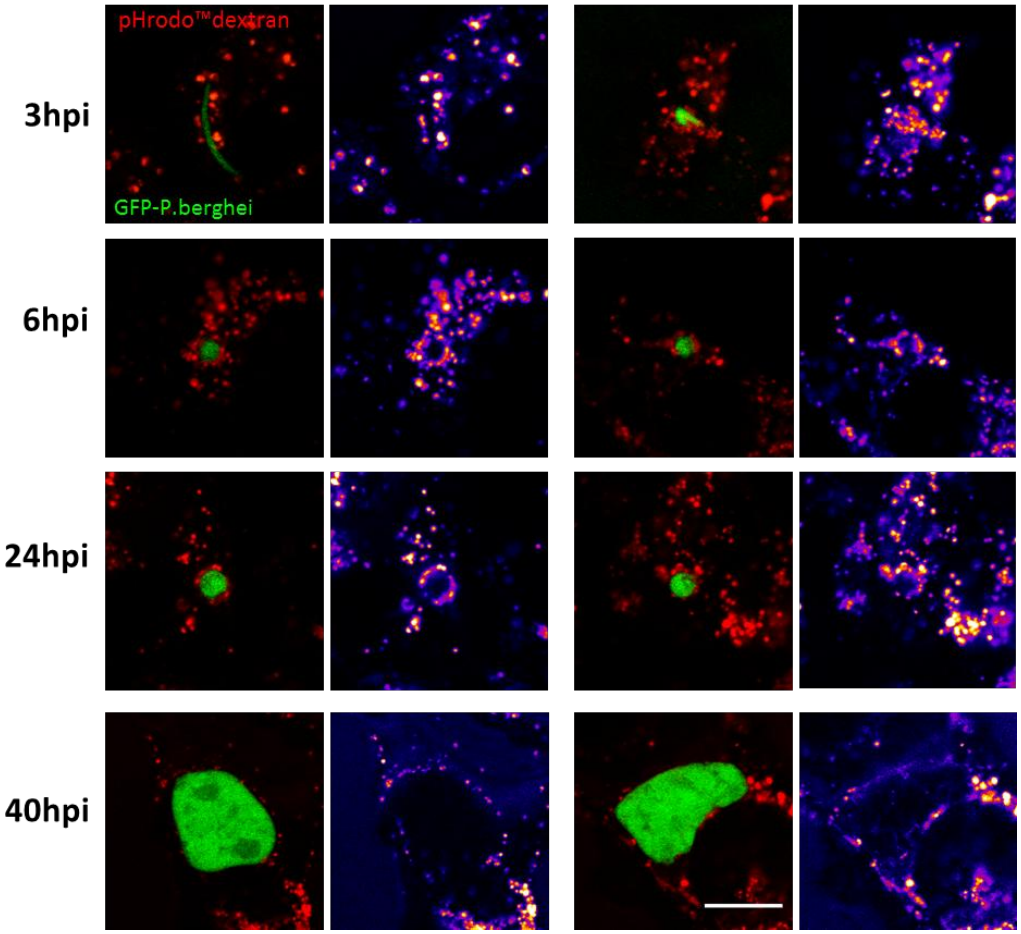


Figure 4.4 - P.berghei PVM is surrounded by acidic vesicles but maintains a neutral pH. For visualization after 3 and 6 hours post infection, cells were incubated with pHrodo™dextran (red and pseudocolored in right image) overnight prior to washing and infection with GFP-*P.berghei* (green). To visualize 24 and 40 hours post infection, cells where infected with GFP-P.*berghei* parasites and later incubated with pHrodo™dextran as described. pHrodo™dextran images are pseudocoloured so that less intense vesicles (more neutral) are purple and more intense vesicles (more acidic) are shown in pink and yellow. Scale bar: 10µm.

Figure 4.4 shows GFP-*P.berghei* parasites at various times post infection in cells incubated with pHrodo™dextran, showing acidic vesicles in close proximity to the parasite PVM, in a punctate staining pattern. This is very different from the continuous fluorescent rim observed when the same cells were fed latex beads, where fusion with acidic lysosomes occurs (**Figure 4.3**). Importantly, and as seen with LysoTracker®Red, pHrodo™dextran does not stain inside the parasite vacuole, indicating that the internal vacuolar space between the parasite membrane and the PVM is not acidic.

Furthermore, since pHrodo™dextran allows for a relative measurement of the acidity of the stained vesicles, it is possible to notice that, although very heterogeneous, the vesicles found surrounding the parasite are mostly yellow, indicating that they have a low pH relative to the rest of the vesicles in the cell.

Taken together, these results suggest that although acidic vesicles are seen in the parasite vicinity, no evidence of fusion with the PVM was observed. Alternatively, some fusion between these vesicles and the PVM may occur but parasites are able to buffer the pH within the vacuolar space. To further characterize the identity of these vesicles, infected cells were stained with an anti-Cathepsin D antibody, an abundant aspartic protease present only on mature degradative lysosomes (Zaidi et al., 2008).

4.2 *Plasmodium* is not surrounded by Cathepsin D positive vesicles

A key feature of competent mature lysosomes is the presence of degredative enzymes, such as lysosomal proteases, nucleases and hydrolases. One of the most abundant is Cathepsin D, an aspartic protease of the pepsin family (Zaidi et al., 2008). Cathepsin D is involved in various cell processes such as protein degradation, apoptosis and autophagic organelle degradation (Benes et al., 2008). Infected cells were stained with a polyclonal antibody that recognizes both active Cathepsin D (46 kD) and its precursor (54kD) isolated from human liver. Other antibodies recognizing mouse Cathepsin D were

tested with no success, and so, in this experiment, a human hepatoma cell line (HUH-7) was used. **Figure 4.5** shows representative images of parasites, stained with 2E6 (green) and anti-Cathepsin D antibody (red) at various stages of liver development.

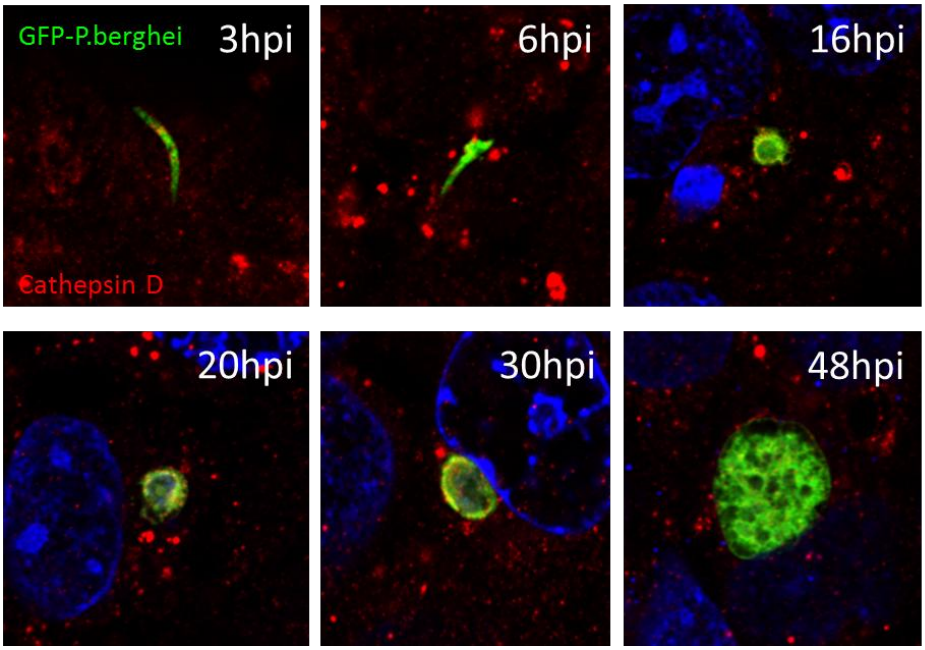


Figure 4.5 – Cathepsin D positive vesicles do not aggregate around P.berghei parasites. Hepa1-6 cells were infected with *P.berghei* parasites and samples were fixed at various times post infection. Samples were stained with anti-Cathepsin D antibody (red) and parasites were stained with 2E6 (green). Nuclei were stained with DRAQ5 (blue). Scale bar: 10µm.

Figure 4.5 shows that *P.berghei* parasites are not surrounded by Cathepsin D positive vesicles. This could exclude mature hydrolytic degradative acidic lysosomes as the vesicles seen aggregating around parasites although other enzymes, such as Cathepsin L, known to be present in mature hydrolytic lysosomes could be present.

In order to further investigate the role of these vesicles in *Plasmodium* infection, we proceeded to disrupt or interfere with the trafficking and fusion of these vesicles and analyze its effect during Plasmodium liver infection. Since an siRNA targeting approach did not yield conclusive results, we proceeded to

use a pharmacological approach, where drugs known to affect the lysosomal pathway were used to study the role of these vesicles in *Plasmodium* liver infection.

4.3 Disrupting vesicle function – a pharmacological approach

Many drugs have been shown to have an effect on lysosome biogenesis, maintenance and fusion with other cellular vesicles. A key aspect of lysosomes is their low internal pH which is instrumental for their function (van Deurs et al., 1996). Thus, one method to disrupt lysosome integrity is to interfere with lysosome pH.

The vATPase proton pump that acidifies the lysosomal lumen consists of a V1 complex that hydrolyses ATP and transfers energy to the membrane-embedded V0 complex that translocate H^+ across the bilayer (Beyenbach and Wieczorek, 2006). Lysosome acidification is not only a consequence of lysosomal formation but seems to be an integral part of the lysosome maturation process, as it directly controls membrane traffic (Gordon et al., 1980)(van Deurs et al., 1996).

Studies affecting lysosome function often use one of two methods; dissipation of the pH gradient across lysosomes, arresting maturation and redistributing lysosomes, achieved using a weak base such as ammonium chloride (NH_4Cl) (Gordon et al., 1980), or by inhibiting the V-ATPase pump using drugs such as Concanamycin A (Dröse and Altendorf, 1997)(Sobota et al., 2009). These treatments were also described to inhibit autophagosome-lysosome fusion (Yamamoto et al., 1998)(Kawai et al., 2007), inhibit the transfer of material from late endosomes to lysosomes (Mousavi et al., 2001) although their primary function is the inhibition of intralysosomal degradation due to the increased internal pH (Klionsky et al., 2008).

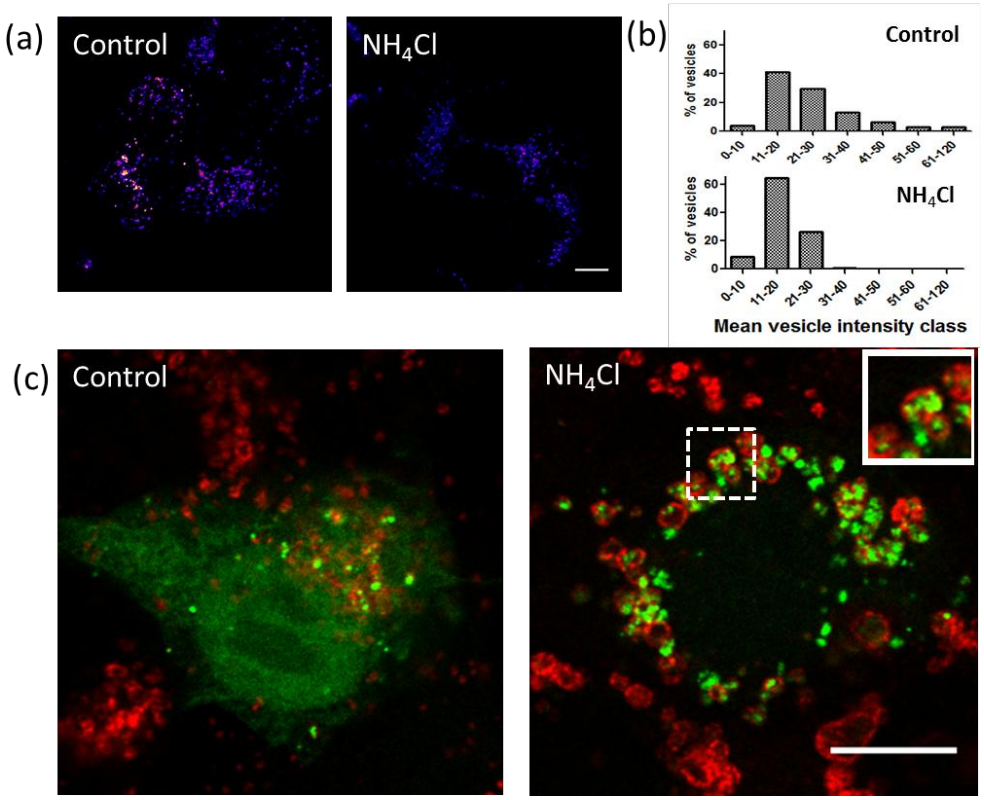


Figure 4.6 – NH_4Cl treatment inhibits acidification in Hepa1-6 cells and effects lysosome trafficking. (a) Hepa1-6 cells were incubated with 25mM NH_4Cl for 16 hours and stained with pHrodo™dextran (pseudocoloured image where less intense vesicles (more neutral) are purple and more intense vesicles (more acidic) are shown in pink and yellow). Scale bar: 10µm. (b) Quantification of the mean intensity of the vesicles seen in image (a) in control and NH_4Cl treated cells. (c) Hepa1-6 cells were transduced with GFP-LC3 virus (green), incubated with NH_4Cl for 16 hours, fixed and stained with anti-LAMP1 antibody (red). Scale bar: 10µm.

Incubating Hepa1-6 cells with 25mM NH_4Cl for 16 hours raised the pH and decreased the number of acidic vesicles, as shown in **Figure 4.6**. When treated cells were also incubated with pHrodo™dextran, the mean intensity of stained vesicles was significantly lowered when compared to untreated control cells, indicating that the pH of these vesicles was effectively increased. Note that the quantification of NH_4CL treated vesicles is a large overestimation, since vesicles that are not sufficiently acidic will not even be stained with pHrodo™dextran and thus are not accounted for in this quantification. When cells treated with NH_4Cl were stained with anti-LAMP1 antibody, late

endosome/ lysosome morphology was severely affected, showing a generalized swelling of this compartment (vacuolation) (shown in red). Furthermore, when cells were transduced with GFP-LC3 adenovirus, a construct where the GFP fluorescent signal is quenched in low pH vesicles (Kabeya et al., 2000)(Bampton et al., 2005)(Mizushima et al., 2010), and stained with anti-LAMP1 antibody (**Figure 4.6**), these enlarged vesicles had smaller GFP-LC3 vesicles inside (see inset), indicating that the pH of these abnormal organelles was effectively raised and that proper GFP protein degradation was inhibited. In untreated cells, GFP-LC3 is present throughout the cytosol and is concentrated in puncta which do not colocalize with LAMP1 vesicles, as expected.

It is also important to note that there is a significant increase in the amount of GFP-LC3 signal in treated cells although this does not necessarily mean that autophagy is up-regulated, since it is protein degradation that is being inhibited in these vesicles due to the lack of acidic functionally degradative vesicles.

In order to study the effect of NH_4Cl on parasite infection, cells were infected with parasites and the drug was added one hour post infection, to avoid affecting parasite migration and invasion. As seen in **Figure 4.7**, when infected cells were incubated with 25mM NH_4Cl for the initial 16 hours of infection, parasite infection rate was not significantly affected compared to non-treated control cells, when measured at 16 hour post infection. Curiously mean parasite size decreased slightly (mean size of 11.22μm^2 in control vs 8.72 μm^2 in treated cells, P-value 0.0036). A similar result was observed when parasites were treated with NH_4Cl during the replication phase of parasite development, from 16 to 40 hours post infection. Again, only EEF size was affected, decreasing almost three fold in treated cells (mean size of 96.19μm^2 in control vs 30.71μm^2 in treated cells, P-value 0.0001).

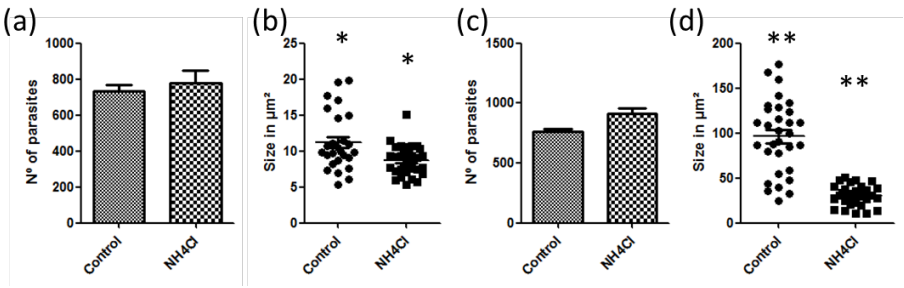


Figure 4.7 – Inhibition of acidification using NH_4Cl effects P.berghei growth during the late stages of infection. Hepa1-6 cells were infected with *P.berghei* parasites and (a)/(b) one hours post infection, treated with NH_4Cl. Infection was stopped at 16 hours post infection. (c)/(d) Cells were infected and incubated treated with NH_4Cl at 16 hours post infection. Infection was stopped at 40 hours post infection. (a)/(c)Total number of parasites and (b)/(d) the size of EEFs was quantified. *P-value: 0.0036; **P-value: 0.0001.

In order to confirm these results, Concanamycin A, a known inhibitor of the vATPase proton pump, was used to block vesicle acidification (Dröse and Altendorf, 1997)(Sobota et al., 2009). As described in Sobota et al, cells treated with Concanamycin A showed the same enlarged LAMP1 vesicles (**Figure 4.8**) indicating that the drug was effective.

Cells treated with 0.1nM Concanamycin A during the initial 16 hours of infection showed no effect on parasite infection rates and size (**Figure 4.8**), similar to the results obtained with NH_4Cl treatment. When cells were treated from 16 to 40 hours post infection, mean EEF size was significantly decreased, although less dramatically than in NH_4Cl treated cells (mean size of 96.19µm^2 in control vs 53.65µm^2 in treated cells, P-value 0.0036). Another vATPase inhibitor, Bafilomycin A (van Deurs et al., 1996), was also tested but cells incubated for long periods with this drug showed signs of stress and apoptosis and thus was not used in the context of *Plasmodium* liver infection.

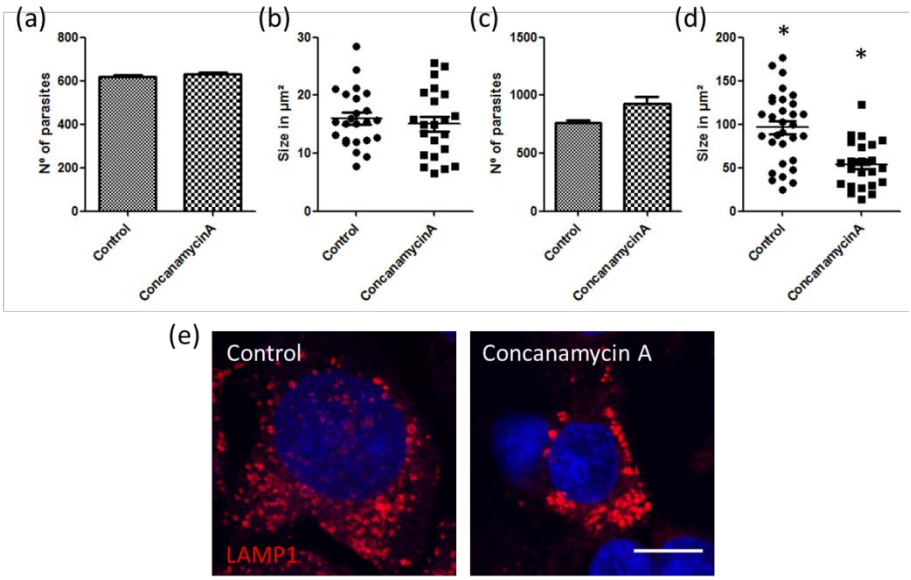


Figure 4.8 - – Inhibition of acidification using Concanamycin A effects P.berghei growth during the late stages of infection. Hepa1-6 cells were infected with *P.berghei* parasites and (a-b) one hours post infection, treated with Concanamycin A. Infection was stopped at 16 hours post infection. (c-d) Cells were infected and incubated with Concanamycin A at 16 hours post infection. Infection was stopped at 40 hours post infection. (a and c)Total number of parasites and (b and d) the size of EEFs was quantified. *P-value: 0.0001. (e) Hepa1-6 cells were incubated with Concanamycin A for 16 hours, fixed and stained with anti-LAMP1 antibody (red) and DRAQ5 for the nuclei (blue). Scale bar: 10µm.

Taken together, these studies suggest that acidic vesicles may be playing a role in parasite nutrient acquisition, since when the trafficking of these vesicles is impaired, EEF size decreased. However, for this exchange of nutrients, lipids and/or membrane to occur, some vesicles fusion, and/or contents discharge from these vesicles to the PVM space would have to occur. Because light imaging techniques do not have the necessary resolution to visualize this, transmission electron microscopy samples were prepared and analyzed.

4.4 Plasmodium parasites (may) feed on host vesicular structures

Based on conventional morphological identification, various types of host vesicles were seen in the parasite periphery, often almost touching the PVM (**Figure 4.9**). Samples were prepared and serial sections were analyzed. Although the observation of a fusion event was very unlikely, many of these host vesicles were seen within just a few nanometers of the parasite PVM (**Figure 4.9**), and some material was even seen in the PVM space (**Figure 4.10**), both suggesting that fusion between these two membranes could occur and that these vesicles could be a source of nutrients for growing parasites.

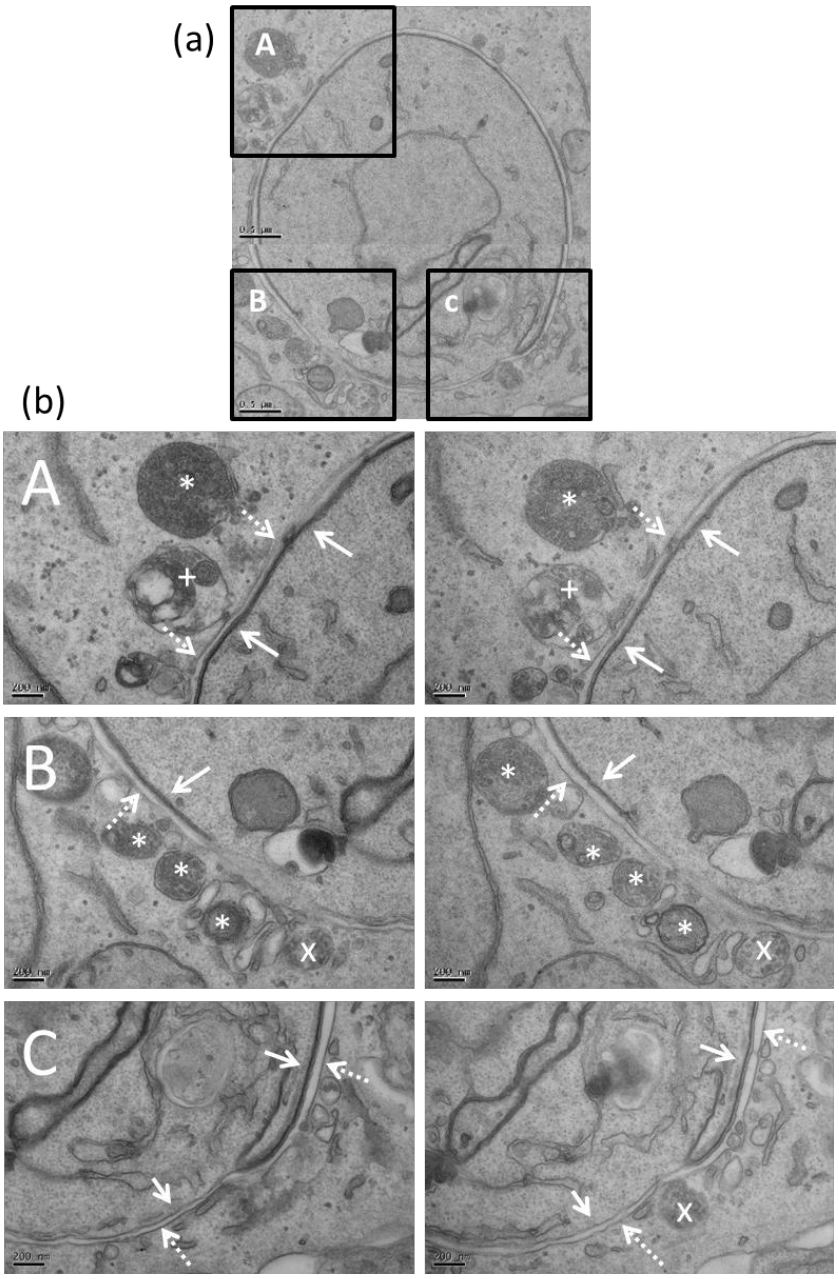


Figure 4.9 - Transmission electron microscopy images suggest close interaction between P.berghei PVM and host vesicles. Hepa1-6 cells were infected and fixed at 24 hours post infection. Samples were prepared for electron microscopy analysis. (a) An entire parasite cross-section is seen with various host vesicles surround the PVM. Scale bar: 0.5µm. (b) insets shown in (a) were magnified and 2 serial sections are shown. Solid arrows point to the parasite membrane, dashes arrows point to the PVM, (*) indicate host vesicles which resemble lysosomes, (x) indicate possible host MVBs and (+) indicate possible host autophagosomes. Scale bar: 200nm.

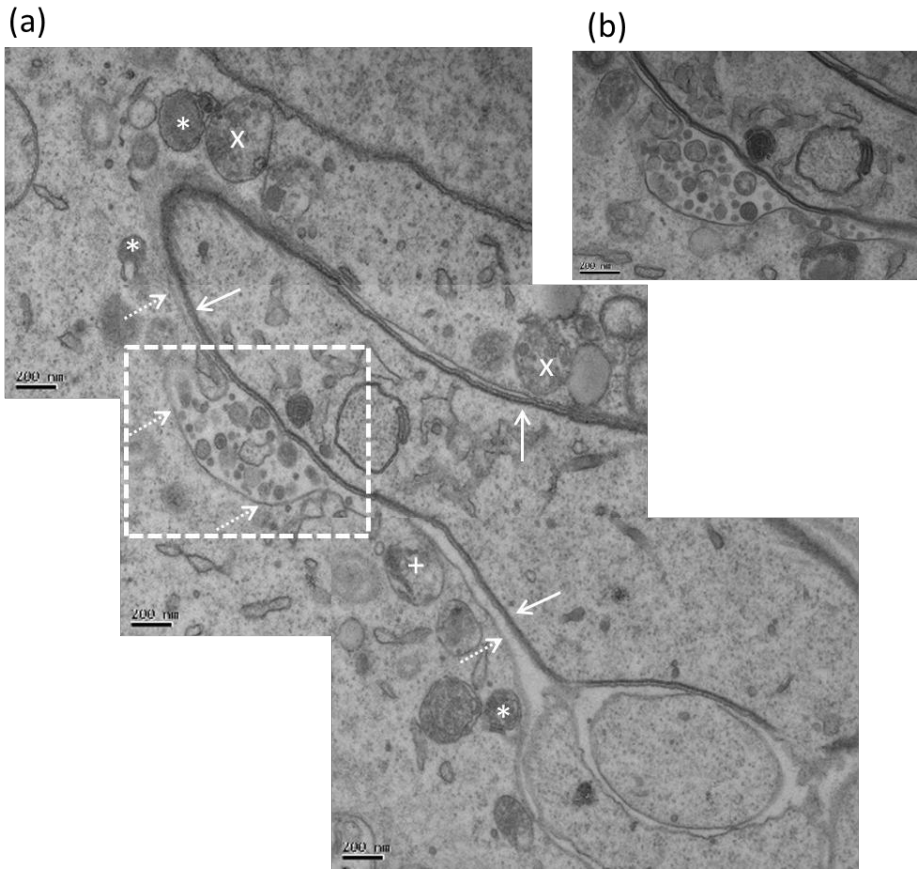


Figure 4.10 - Transmission electron microscopy images suggest exchange of vesicle contents between host vesicles and the P.berghei PVM. Hepa1-6 cells were infected and fixed at 24 hours post infection. Samples were prepared for electron microscopy analysis. (a) An entire parasite cross-section is seen with various host vesicles surround the PVM Solid arrows point to the parasite membrane, dashes arrows point to the PVM, (*) indicate host vesicles which resemble lysosomes, (+) indicate possible host autophagosomes. Scale bar: 200nm. (b) A different serial section of the insets shown in (a) where small vesicles are seen in the vacuolar space. Scale bar: 200nm.

Although morphological analysis alone does not point towards the identity of the vesicles that aggregate around parasite, it has allowed the observation that these vesicles are found only a few nanometres away from the PVM, suggesting that they are close enough for fusion between the two membranes to occur. Further EM studies, such as cryo-immuno EM, using different membrane markers may help to further elucidate the characteristics of these vesicles and if they fuse and are present on the parasite PVM.

From the results shown in this Chapter, it seems that at *Plasmodium* parasites are surrounded by host vesicles which have the membrane markers Rab7a/CD63/LAMP1 and are acidic. From the results obtained in the previous Chapter, vesicles from the autophagic pathway, containing the membrane marker LC3 were also observed around parasites throughout the entire infection. In order to investigate if the LAMP1 vesicles were also LC3 positive, suggesting that these are autophagosomes which has already fused with late endosomes/MVBs, termed amphisomes, Hepa1-6 were transduced with GFP-LC3, infected with *P.berghei* parasites and stained with anti-LAMP1 antibody. As shown in **Figure 4.11**, some of the LAMP1 positive vesicles around parasites are also LC3 positive, suggesting that these vesicles may be amphisomes.

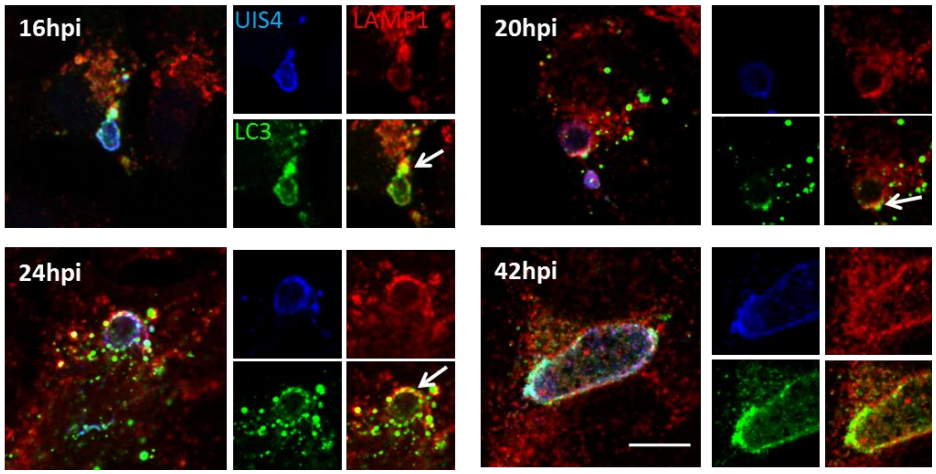


Figure 4.11 – LAMP1 colocalizes with autophagic vesicles which aggregate around the parasite. Hepa1-6 cells were transduced with GFP-LC3 (green) 24 hours prior to infection with *P.berghei* sporozoites. Cells were fixed at various times post infection and stained with anti-UIS4 antibody (blue) to stain the parasite PVM and anti-LAMP1 antibody (red). Arrows indicate LAMP1/LC3 positive vesicles, shown in yellow (arrow). Scale bar: 10µm.

4.5 Discussion

According to the results shown so far, *Plasmodium* parasites are surrounded by host vesicles which show a close association with the parasite PVM. A series of membrane markers, such as Rab7a, CD63, LAMP1 (all present on late endosomes/ multivesicular bodies (MVBs) and lysosomes) as well as the autophagic marker, LC3, which may be found on autophagosomes and amphisomes, are all found aggregating around parasites throughout the liver infection.

Using two independent dyes which specifically concentrate and fluoresce in acidic vesicles, parasites were shown to be surrounded by vesicles with a low pH, although their intravacuolar space was never acidified. This result would suggest that either fusion between the highly acidic lysosomes found on the parasite periphery is inhibited or, alternatively, parasites allow fusion with these vesicles but are capable of buffering the pH within the vacuolar space, in order to avoid degradation. In order to distinguish between these two hypotheses, immuno-electron microscopy techniques could be used to try and show that the membrane markers found on the vesicles aggregating around parasites are also found in the PVM, indicating fusion between the two membranes. Unfortunately, this was not done in the context of this study but is being further investigated in the lab.

Along with the low pH, another important feature of degradative lysosomes is the presence of hydrolytic enzymes, such as Cathepsin D. Surprisingly, Cathepsin D positive vesicles were never seen surrounding parasites in HUH7 cells. Curiously, these cells only had a small number of Cathepsin D positive vesicles, less than expected. Cathepsin D is only one of the many hydrolytic enzymes shown to be present in lysosomes, so it could be possible that HUH7 cells are more abundant in other degradative enzymes, such as Cathepsin L. Thus the possibility that parasites may be surrounded by hydrolytic degradative vesicles cannot be discarded at this point.

In order to further investigate the role of these vesicles in *Plasmodium* infection, we proceeded to disrupt or interfere with the trafficking and fusion of these vesicles and analyze the effect during *Plasmodium* liver infection. Studies using ammonium chloride and Concanamycin A, which inhibit vesicle acidification had no effect on *Plasmodium* infection rate but had a surprising effect on EEF size, decreasing it almost threefold at 40 hours post infection.

Taken together, these studies suggest that when the pH of cells is increased, acidic vesicle trafficking and fusion is affected and lysosome maturation is inhibited. As a consequence, late endosome, MVB, amphisomes and lysosome formation and trafficking is inhibited, since these vesicles are all acidic in normal conditions (<pH5.5) (Casey et al., 2010). Of these vesicles, only amphisomes have the LC3 protein marker, suggesting that these vesicles may have a prominent role in *Plasmodium* development. Since EEF size was reduced when these vesicles were impaired, this suggests a novel role for these organelles in parasite growth and could be a source of nutrients and lipids for the growing intracellular parasite, especially during the later stages of infection.

These results correlate well with the data obtained with Rab1a, since Rab1a downregulation decreased EEF size as measured at 40 hours post infection. Since Rab1a is involved in autophagosome production, cells with depleted levels of Rab1a have the autophagic pathway at least partially inhibited, decreasing the pool of available amphisomes for parasite to feed upon, effectively decreasing their size compared to control cells (see **Figure 3.4**). The opposite happened when Rab1a was overexpressed in cells (**Figure 3.6**).

5. General Discussion

Approximately half of the world´s population is at risk from malaria. This deadly disease is one of the most prevalent causes of morbidity and mortality in endemic countries. Although the widespread implementation of intervention programs has diminished the burden of malaria in many places, unless these programs are adequately maintained for long periods of time and a large geographical area, making them extremely expensive, new malaria infections will ultimately reappear.

Current anti-malarial drugs, if taken properly, are extremely effective at preventing human deaths, but these drugs cannot be taken for prolonged periods as they are highly toxic and facilitate the emergence and spread of drug resistant strains (Greenwood, 2010). While the development of new drugs is continually explored, especially by large pharmaceutical companies, this is a very slow process, with very high costs. As there is still much that is unknown about the host-parasite interaction, basic research can still have a fundamental role in unraveling new potential targets for effective therapy.

As with all other host-pathogens relationships, the molecular interactions that occur between the host and the invading pathogen are critical for a successful infection. As the development of *Plasmodium* sporozoites inside hepatocytes is an obligatory step in the mammalian life cycle, a better understanding of the cellular interactions required during this stage of the parasite's life could lead to the development of new prophylactic approaches against this deadly parasite.

The work developed in this PhD thesis aimed to dissect some of these host-parasite interactions during *Plasmodium berghei* liver stage development.

Many intercellular pathogens, while establishing an effective infection, need to interact with the host cell to acquire nutrients essential for growth. Pathogens have evolved a multitude of strategies to accomplish this (reviewed in Chapter 1.8). The fact that *Plasmodium* parasites quickly outgrow their host cell hepatocyte *in vivo*, before moving on to the next stage of infection, led us to hypothesize that parasites obtain these essential nutrients, lipids and membranes from the host cell. Thus, the initial part of this project involved

characterizing any possible interactions between the PVM and host endomembrane system.

As previously observed by Bano et al (2007), the host ER was seen surrounding liver stage *Plasmodium* parasites. Parasites, or the extensions of the PVM, were always found in close proximity to the Golgi apparatus, suggesting that an interaction with either of these organelles could occur. From the parasite´s point of view, residing close to either of these two organelles could permit an abundant source of proteins and nutrients, since these two organelles are at the centre of the cell´s biosynthetic pathway.

Interestingly, some intracellular pathogens, such as *Legionella pneumophila* are able to take advantage of the host ER by transforming its vacuolar membrane into an ER-like compartment within 5 minutes of parasite invasion (Tilney et al., 2001). Once inside the ER lumen, these bacteria reside in an organelle rich in peptides and lipids, which could support bacterial growth. *Brucella*, another bacterial pathogens, has evolved a similar strategy as it also replicates within the host ER (Pizarro-Cerdá et al., 1998) suggesting that this is a desirable organelle for growing intracellular pathogens.

Chlamydiae are membrane bound bacteria that acquire nutrients from the host cell by redirecting host transport vesicles and hijack intracellular organelles using a variety of different methods (Saka and Valdivia, 2010). Interestingly, *Chlamydiae* are able to proteolytically mediate fragmentation of the host Golgi apparatus by recruiting Rab14 to its membrane, facilitating the delivery of sphingolipids from the Golgi to the chlamydial inclusions (Capmany and Damiani, 2010). This observation also shows that the Golgi could be an excellent source of nutrients for developing parasites, although the role of this organelle as a source of nutrients during *Plasmodium* liver infection remains to be elucidated.

We proceeded to analyze possible interactions between the host endocytic pathway and *Plasmodium* parasites. No obvious association between early and recycling endosomes and parasites was observed. It cannot be excluded that a very transient association may occur, especially during the

early minutes of infection, as happens with *Brucella* bacteria (Pizarro-Cerdá et al., 1998) although this analysis, as explained before, is difficult to perform using *Plasmodium* parasites.

Despite Bano et al (2007) commenting that "no major alteration in the organization of host lysosomal distribution was observed in *P.berghei* infected cells at 24h post infection" although no data was shown, we proceeded to analyze the position of host late endosomes and lysosomes relative to *Plasmodium* parasites. Surprisingly, when late endosomes/lysosomes were stained using various markers such as Rab7a, CD63 and LAMP1, a very strong aggregation of these vesicles with the PVM was clearly observed.

This prompted us to question the role of these vesicles during *Plasmodium* infection. Due to the acidic nature of these vesicles, their fusion with the PVM could lead to parasite degradation, thus we hypothesized at this point that either the parasite is able to inhibit fusion of these host vesicles with its PVM, by some yet unknown mechanism, or allows some fusion with these vesicles but is able to buffer the possible changes in pH within the intravacuolar space. We therefore proceeded to try and disrupt these vesicles surrounding parasites and observe the effect on *Plasmodium* liver development.

After interfering with various proteins described to be involved in late endosome/ lysosome trafficking with no effect on parasite infection, we proceeded to perform a small scale siRNA screen of Rab proteins, a family of proteins involved in membrane traffic, and investigate their role during *Plasmodium* infection.

Surprisingly, only one protein, Rab1a had an effect on parasite infection rate. As expected, other Rab protein which had previously been tested, such as Rab7a, Rab9a and Rab32, had no effect of parasite infection rate. As discussed before, siRNA technology has many problems, especially since negative results are often inconclusive. Nevertheless, and after studies confirming the role of Rab1a, we were confident that at the time point measured, Rab1a, of all the Rab proteins tested, was having a significant effect on parasite infection rate.

From the colocalization studies performed, a few other Rab proteins showed a possible interesting interaction with *Plasmodium* parasites. Curiously, a number of Rab proteins seen aggregating at some level around parasites, such as Rab8a, Rab8b, and Rab43, have been described to be involved in aspects of Golgi trafficking and maintenance. Together with Rab1a, which is also involved in Golgi trafficking, and the initial results where parasites were seen close to the host Golgi, these results point towards a possible Golgi-parasite interaction during liver infection. These results are being further characterized in the lab.

Since the only functional hit, obtained in this screen, was Rab1a, we proceeded to further characterize the role of this protein during *Plasmodium* infection. The effect of Rab1a depletion throughout liver infection was analyzed and infection rate as well as EEF size was quantified in order to establish if Rab1a was effecting parasite survival or development. As expected, parasite infection rate was doubled during the initial hours of infection in Rab1a depleted cells compared to control, but surprisingly, EEF size was affected only during the later stages of infection. This would suggest that host Rab1a is involved in early parasite death but is also involved in parasite development during late infection.

Since Rab1a has been implicated in both ER-to-Golgi trafficking as well as the autophagic pathway, we investigated which of these two pathways was having an effect on *Plasmodium* liver infection. According to the results presented in this thesis, together with preliminary data from our lab, all evidence points towards the autophagic pathway as having a prominent role during parasite infection. As with Rab1a, autophagy seems to be playing a dual role, mediating parasite death during the initial stages of infection, and having a role in parasite development during the late stages of infection.

As discussed before, various intracellular pathogens have evolved mechanism to avoid interaction with the autophagic pathway, evading autophagic mediated killing (Subauste, 2009). However, other pathogens have evolved strategies not only to interact with this pathway, but to exploit it for

their own benefit. This is the case with *Coxiella burmetii*, a bacterium which not only fuses with vesicles from the autophagic pathway but thrives within phagolysosomes (Berón et al., 2002)(Romano et al., 2007).

More recently, *Toxoplasma gondii* was shown to induce autophagy in host cells, and this contributed to parasite growth (Wang et al., 2009). These results suggest that *T.gondii* parasites, a very close relative of *Plasmodium* parasites, are able to exploit the host cell´s autophagic pathway for nutritive benefits (Orlofsky, 2009). Since *T.gondii* parasites require host cell nutrients, including amino acids, lipids and purines for growth (Fox et al., 2004)(Mazumdar and Striepen, 2007)(Ghérardi and Sarciron, 2007), the host autophagic pathway which results in nutrient rich lysosomes, could be utilized by parasites. According to the results presented in this work, a similar mechanism could be used by *Plasmodium* parasites during liver stage development, although the exact mechanism by which these two parasites are able to uptake these vesicles without being destroyed in the process remains unknown.

Further studies (shown in Chapter 4) also showed that the vesicles surrounding parasites are acidic, although the parasite vacuolar space maintains a neutral pH. This suggests that if these acidic vesicles are indeed fusing with the PVM, the parasite is able to buffer the changes in pH preceding this fusion. In order to observe any possible fusion events, electron microscopy images were produced and analyzed. Since the usual infection rates observed with *P.berghei* parasites are around 2-5%, not many parasite were found, which reduced the probability of finding an infected cell and therefore a fusion event, which are usually very transient. Nevertheless, as seen in **Figure 4.9** and **Figure 4.10**, various host vesicles, which morphologically could to be multivesicular bodies or amphisomes, since they are membrane bound vesicles with heterogeneous material inside, were seen very close to the PVM. It would be very interesting to perform immuno-electron microscopy, using the proteins already analyzed by immunofluorescence and test if they are present on these

vesicles and on the PVM itself, it order to verify if fusion between these two membrane occurs.

Interestingly, other intracellular pathogens are able to interact with host lysosomes in different ways. For example, while *Legionella* avoids interaction with host lysosomes, *Brucella* is seen surrounded by LAMP1 positive vesicles and also have the autophagic marker monodansylcadaverin around them, while mannose 6-phosphate receptors and Cathepsin D vesicles are absent from the parasite vicinity (Pizarro-Cerdá et al., 1998) suggesting a highly complex mechanism of host cell subversion by *Brucella* bacteria.

Drugs known to disrupt or interfere with the trafficking and fusion of vesicles within the endocytic/autophagic pathway were used to try and unravel the role of these vesicles during *Plasmodium* infection. When the pH of host vesicles was increased, using ammonium chloride or Concanamycin A, both treatments had no effect on parasite infection rates while parasite size was impaired during the later stages of infection. This would suggest that, like the results obtained with Rab1a, these vesicles are involved in parasite growth and development.

It was expected that, by inhibiting acidic vesicle trafficking, parasite infection rates would increase, assuming that these vesicles are involved in parasite clearance during early infection. Surprisingly, this was not the case. The only treatments that had an effect on parasite survival during the first hours of infection were when cells were depleted for either Rab1a or VPS34 (preliminary results by Laura Santos). Since both these proteins are involved in early autophagosomes formation, it is possible that autophagosomes are involved in early parasite elimination. Autophagosomes are not normally acidic and so treatment with ammonium chloride and Concanamycin A would not affect their trafficking. Although autophagosomes have never been described to date as being able to degrade pathogens, this hypothesis cannot be ruled out at the present time and would explain a pH independent pathway used by cells for killing pathogens.

This hypothesis would correlate well with the results obtained with cells treated depleted for Rab7a (Chapter 2), which had no effect on parasite numbers. Since Rab7a has been implicated in the fusion of late endosomes with autophagosomes (Gutierrez et al., 2004)(Jäger et al., 2004) and not with autophagosomes formation, and thus autophagosomes number would not be altered in these cells. This would explain why parasites numbers are not affected by Rab7a downregulation, if indeed it is autophagosomes that are involved in parasite elimination during the early stages of *Plasmodium* infection.

Taken together, the data presented in this thesis points towards a new role of host vesicles, from the autophagic pathway, as an important source of nutrients for parasites liver development and possibly for parasite elimination during the initial stages of *P.berghei* infection in the liver. Since some parasites are able to survive past these initial stages, and develop to form fully mature merozoites, this implies that parasites are able to withstand this cell mediated killing and establish a fruitful host-parasite interaction throughout the remainder of the infection in the hepatocyte. The host protein Rab1a was also identified as important host factor during hepatocyte infection, possibly due to its role in the host autophagic pathway.

In conclusion, this study provided a crucial basis for more extensive studies required to better understand the role of the host-parasite interactions described as well as to identify new ones. Understanding the *Plasmodium* liver infection is crucial for the development of novel prophylactic measures to combat this disease. With increased knowledge of the molecular players involved underlying parasite invasion and development in the mammalian host, one day it may be possible to target this human pathogen, without the serious side effects which occur with the current drugs.

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Supplementary Data

Supplementary Figure 1

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Supplementary Table 1

List of siRNA genes screened and their specific sequences, as well as their Dharmacon identification code.

Well name	Gene Symbol	NCBI Accession Number	GI Number	Gene Id	Pool Number	siRNA Duplex 1	siRNA Duplex 2	siRNA Duplex 3	siRNA Duplex 4
B02	siControl				D-001206-13				
B03	siControl				D-001206-13				
B04	siControl				D-001206-13				
B05	siControl				D-001206-13				
B06	siControl				D-001206-13				
B07	siControl				D-001206-13				
B08	siControl				D-001206-13				
B09	siControl				D-001206-13				
B10	siControl				D-001206-13				
B11	siControl				D-001206-13				
C02	Praf2	NM_138602	20070421	54637	M-042962-00	UCAACAACCUCCUCUACUA	CCAUUAGCCUCUUCAUUCU	GAUCGAGAGCAUCGGUCUA	GGGAUUGCUUCUAGAGGCA
C03	Chml	NM_021350	10946669	12663	M-047844-00	GUUCAAACGUGCAAUAUAA	AGCCGGAGAUUUAAUAUUG	UACAUUAGAUGGCCUUCAA	GCGAUAAGACGGAUAUGCA
C04	Ggps1	NM_010282	47271502	14593	M-047540-00	GCUAAAGCCUACAAACAAA	GUAAGAUGUUCACAGAAGA	UGGGAACCCUUCACUAGUG	GGAUUCUUCUAGAGCCCUA
C05	Fdps	NM_134469	31982573	110196	M-059452-00	CGACUACCUUGAUCUCUUU	UAGAACUGCUCCAGGCUUU	CAACAGCGCCAGAUCUUAG	CGGGAAAGGUCGGCACUGA
C06	RabGGTB	NM_011231	6755263	19352	M-043799-00	GGGAAUAAGUGCUAGCAUU	UGGAGAAGCAUGCCGAUUA	UAGAUAAGGUCGUGGCCUA	GAGAAUGAGCGGCGUCUAU
C07	Rab1a	NM_008996	31543563	19324	M-040850-00	GAACAAUCACUUCCAGUUA	GCCGAGAAGUCCAAUGUUA	GAUGAUACGUAUACGGAAA	CAGCAUGAAUCCCGAAUAU
C08	Rab1b	NM_029576	21313161	76308	M-050441-00	CGAAUAUGACUACCUGUUU	CCAAGAAUGCCACCAAUGU	GUGAGAAUGUCAAUAAACU	GAACGGCCCAACCUGAAGA
C09	Rab2	NM_021518	31543566	59021	M-040851-00	GGACUUAUCUUCAUGGAAA	GUAGAGGAGGCAUUUAUUA	GAAGGGGUCUUUGACAUUA	CACAAUUGGUGUAGAGUUU
C10	Rab2b	NM_172601	30525050	76338	M-055076-00	CAGCAAGGUUUAUUUGAUG	GACUUACGCUUAUCUCUUC	GAGCACGUAUGGUCAACAU	CACCUGACCUCAUGGUUAG
C11	Rab3a	NM_009001	31560641	19339	M-060407-00	CCACUCAGAUCAAAACUUA	UGUCAGCACCGUUGGCAUA	GGACGUGAUCUGUGAGAAG	CCAUCUACCGCAACGACAA
D02	Rab3b	NM_023537	31543569	69908	M-059841-00	CUACUCAGAUUAAGACCUA	AAAGAGUGGUCCCAACUGA	GCGAUAAGAUGUCUGACUC	UGGUAAGACUGGAAUCAAA
D03	Rab3c	NM_023852	31560045	67295	M-061032-00	CAACUCAGAUCAAAACAUA	GUAGAUAUCAUUUGUGACA	GGCAUCGAUUUCAAAGUAA	GACGAUUCCUUUACAUCUG
D04	Rab3d	NM_031874	24475731	19340	M-040852-00	GGAUCAAGCUGCAGAUUUG	GGUCGUACCUGCUGAGGAU	CCAAGGAGAAUAUCAAUGU	GUGACAAGAUGAAUGAAUC
D05	Rab4a	NM_009003	6679594	19341	M-040853-00	UGACUCAAAUCAUACCAUA	GACUUACGAUUUCUUGUUU	GGGCACUCCUUGUCUAUGA	AAAUGUCGGUGGUAAAUAU
D06	Rab4b	NM_029391	21313011	19342	M-040854-00	GAGGAUGGGUUCAGGCAUU	CCUAGAGGCUUCUCGCUUU	CAAGAUCGACUCAGGUGAA	GGAAGACUGUGAAACUACA
D07	Rab5a	NM_025887	31981152	271457	M-040855-00	AAACAAAGCUGACUUAGCA	GGUCAAGAACGGUAUCAUA	GCACAAGCAGCCAUAGUUG	GGCCAAAUACUGGAAAUAA
D08	Rab5b	NM_177411	31343093	19344	M-040856-00	GAACGUGAAUGAUCUCUUC	GGAGCGAUACCACAGUUUA	UGAAGAGGCUCAGGCAUAU	UCAAAGGACAGUUCCAUGA
D09	Rab5c	NM_024456	29789256	19345	M-040857-00	CUAAGAAGCUUCCCAAGAA	GCAAUGAACGUGAAUGAAA	AACAAGAUCUGUCAGUUUA	CAACAUCGUCAUUGCACUA
D10	Rab6	NM_024287	31543570	19346	M-040858-00	CCAAAGAGCUGAAUGUUAU	GGAAAGACGUCCUUGAUCA	GGAAUCCGCUGAGGAAAUU	GCAGAGAAGACAUGAUUGA
D11	Rab6b	NM_173781	40254255	270192	M-041645-00	AGACGGAUCUGGCUGAUAA	CAUCGGGAUUGACUUCUUG	CUAAAUGGAUUGAUGACGU	GGUGGUAUAUGACAUUACU

Well name	Gene Symbol	NCBI Accession Number	GI Number	Gene Id	Pool Number	siRNA Duplex 1	siRNA Duplex 2	siRNA Duplex 3	siRNA Duplex 4
E02	Rab7	NM_009005	6679598	19349	M-040859-00	GUACAAAGCCACAAUAGGA	AAACAACAUUCCUUACUUC	AAACAAGAUUGACCUGGAA	AAGUGGAACUGUACAAUGA
E03	Rab7L1	NM_144875	21450114	226422	M-053101-00	UGACACGACUCUACUAUAG	CAAGGGAACUACAUCAAUC	GGACAGCAAGCUCACACUA	GCCGAGAUCACCUGUUUAA
E04	Rab8a	NM_023126	12963498	17274	M-040860-00	GAAUAAGUGUGAUGUGAAU	GAAGACCUGUGUCCUGUUC	GACCUACGAUUACCUGUUC	GAGCAGCCAUGGAGUCAAG
E05	Rab8b	NM_173413	40254271	235442	M-055301-00	GAAGUUAGCAAUUGACUAU	CGAUAGAACUCGACGGAAA	GAGGAGCCAUGGGAAUUAU	GCGAAGACGUACGAUUAUC
E06	Rab9	NM_019773	9790226	56382	M-040861-00	UCACAGAGCUUCCAGAAUU	GAACAGAUAUGUAACCAAU	CAACAAGACUGACAUAAAA	AAACUCAUCUUGCUGUUGA
E07	Rab9b	NM_176971	28892800	319642	M-052946-00	GAAAUUACCCUUAUCUAGA	GGUGUUAGCUGUAGAGGAA	GCUCACUUAUGAACCGUUA	AAAUCGAGAUCUGGAGGUA
E08	Rab10	NM_016676	31543561	19325	M-040862-00	GGAAUAGACUUUAAGAUCA	CAAGAGAGUUGUACCGAAA	CACAUUAGCUGAAGACAUC	UAGAUGAGCAUGCCAAUGA
E09	Rab11a	NM_017382	31980839	53869	M-040863-00	CUAAGCAUCUCACAUAUGA	GUAGGUGCCUUAUUGGUUU	GCAACAAUGUGGUUCCUAU	UGAAAGAACUGAGAGAUCA
E10	Rab11b	NM_008997	6679582	19326	M-040864-00	ACAGAAAUCUACCGUAUUG	CGAGUACGAUUACCUAUUC	GCAGAUAGCAACAUUGUCA	GUGCACUGCUGGUAUAUGA
E11	Rab12	NM_024448	39930444	19328	M-040865-00	GAAAUAAGCUGGACUGUGA	GAAACGAGUUAUCCAAUAG	UGACAAGUAUGCUUCAGAA	UAACCGGGAUGCGCUUCUG
F02	Rab13	NM_026677	21311974	68328	M-045749-00	GCAUUAUCCUCGUAUAUGA	GACAAUAACUACCGCCUAU	GAUCGGGAACCAACAGUAA	GAUCCGAACCGUGGACAUA
F03	Rab14	NM_026697	31982642	68365	M-040866-00	CAACUACUCUUACAUCUUU	ACAGAGAGAUGUUACCUAU	GAGGACGGCUAACCAGUGA	GAAAUAAAGCAGACUUGGA
F04	Rab15	NM_134050	31559980	104886	M-061135-00	GGAAUAAGGCUGAUGAAGA	UCAGUGACGUGGAUGAGUA	CCAUCGGUGUUGACUUUAA	GGUACCAGACUAUCACAAA
F05	Rab17	NM_008998	31543562	19329	M-058833-00	CCUCAUCUCUGAAGCUUGA	AUGCUGCGCUCCUGGUUUA	UGACAUCACUCGGAAGGAU	GAGAAGUACCAAAGCGUCU
F06	Rab18	NM_181070	31341807	19330	M-040867-00	GAAACAUACUGUACAAGAA	UUACUGCUCUGUGCUAUAA	GCAAGCAUUCUAUGUUGUU	AAACUUGCAAUAUGGGAUA
F07	Rab19	NM_011226	33859607	19331	M-042333-00	GAACAUAGAUGAAGUCUUU	ACGCAGCUAUCAUCGCUUA	AUAUGGAGCUGCCAACUUG	GCUCAUGGCGAAGGAGUUA
F08	Rab20	NM_011227	45593125	19332	M-063804-00	CCGCUAUCAUCCUUACAUA	GGUGGUACCUAUGAUCAUG	GCAGUGGCGUUCCUUCAAC	UGCCAAGACUGGAUACAAU
F09	Rab21	NM_024454	33859750	216344	M-048489-00	GCACGUCUCUAUUCAAGAA	GAGUAAACCUUGCCAUAUG	AAACUGGGUCAAAGAAUUA	GAGCCAAGCAUUACCAUAC
F10	Rab22a	NM_024436	21426816	19334	M-054842-00	CAGCAGCCAUCAUCGUUUA	GGGAACAAGUGCGAUCUUA	GAGAUUAGUCGAAGAAUUC	GGAUACGGGUGUGGGUAAA
F11	Rab23	NM_008999	31543564	19335	M-040868-00	GAACAGACACACUCAAGUA	GCAAAGGCAUUUUCACAAA	GAAAGUCGAGCAUGAUUCA	CCACAGACAGGGAAUCUUU
G02	Rab24	NM_009000	31560646	19336	M-040869-00	GAAGAGGGCUGUCAAAUCU	GAAAGUGGCUGAGGAUUAC	UUAAGGAGCUGCGCAGUUU	AAGCUGCAAUUGUCUGUUA
G03	Rab25	NM_016899	31980837	53868	M-040870-00	GAAUCGAACAGAUGAAGAU	AAGAGGGCCUGUUGCAUCA	GGGCACUCCUGGUAUUUGA	UGAGGAGGCCUGCAUGUUU
G04	Rab26	XM_283428	38082074	328778	M-054468-00	CAAAGGAGCUGAAACAGAG	GAAAUUAGCCAAGGAGUAU	GAACAAGGUUGACUCUACU	GGGCAUCGACUUCCGGAAU
G05	Rab27a	NM_023635	31560086	11891	M-060970-00	GGAGAGGUUUCGUAGCUUA	GGAUGGAGAUUACGAUUAC	GAAUCCACCUGCAGUUAUG	AAACAUAAGCCACGCGAUU
G06	Rab27b	NM_030554	13385281	80718	M-050808-00	GCAGGCAAAUGCUUACUGU	CCAGACAUAGUAUUAAUUG	AAACGCUUCUGGACUUAAU	GUCAGAAACUGGAUGAGUC
G07	Rab28	NM_027295	58037190	100972	M-040871-00	GACAAUAGGUGGCAAGAUG	GAGCAUAUGCGAACAGUAA	GAAUGUUACUCUUCAAGUU	GACAGUAAGUGAAGAGUCA
G08	Rab7L1	NM_144875	21450114	226422	M-053101-00	UGACACGACUCUACUAUAG	CAAGGGAACUACAUCAAUC	GGACAGCAAGCUCACACUA	GCCGAGAUCACCUGUUUAA
G09	Rab30	NM_029494	31560777	75985	M-057666-00	CAAUGUAUCGUCACCCUUA	UAAAGUCAUCACUGUGUUA	UCAGCUAUUUGACUUGUUG	AGGCUCAGGACAUGUAUUA
G10	Rab31	NM_133685	19526849	106572	M-051540-00	GGAGUACGCUGAAUCCAUA	GGAUCAAGCUUGGGAAUCA	GGAUUCAUUUCAUACCUUG	GGAAAUCCAGCAUUGUGUG
G11	Rab32	NM_026405	31980965	67844	M-063539-00	CAACAUGACUCGAGUAUAC	AAAUCGACCUGGACAGAAU	CGAUCUGGAUAGUAAAGUU	AAACCUCUGCCAAGGAUAA

Supplementary Table 2

List of Adenovirus constructs produced in this project, including the source of the cDNA, the primer sequences used for cDNA amplification and the restriction sites used for cloning into the pENTR-GFP vector.

Gene	ID #	Source/Cells	Sense #	5´Restriction Site	Sequence	Antisense #	3´Restriction Site	Sequence
Rab1a	240	RAW264.7	641	XhoI	atacctcgagaaatgtccagcatgaatcccgaatatgat	642	KpnI	tacaggtaccaaaagcttctctaggtcactcatcaga
Rab1b	495	RAW264.7	644	EcoRI	atcagaattcatgaaccccgaatatgactacctgttt	645	SalI	gcccgtcgacgctctgaggaatggaggaagaacaagc
Rab2a	525	RAW264.7	650	EcoRI	cgtggaattcatggcgtacgcctatctcttcaagta	651	SalI	actcgtcgacctgtgttttacccaacagcactcatta
Rab2b	538	RAW264.7	647	EcoRI	ttacgaattcatgacttacgcttatctcttcaagta	648	SalI	aactgtcgacgaaaaaggccagcaagccttgcctaag
Rab3a	542	RAW264.7	653	EcoRI	gcatgaattcatgatggcttccgccacagactctcgctat	654	SalI	actcgtcgaccagcaaggtccattcgctttattg
Rab3b	1389	RAW264.7	656	EcoRI	cgtggaattcctcttatccgagatggcttcagt	657	SalI	ttcggtcgacataaacccacctccttgttcagag
Rab3c	558	AtT20	659	EcoRI	atcagaattcatgagacacgaggcgcccatgcagat	660	SalI	ccaggtcgacgatggggtctccaatcaagagcaa
Rab3d	657	RAW264.7	662	EcoRI	attagaattcatggcatccgctagtgagcccct	663	SalI	gctggtcgacccacttccgtttctacacaggatgtaa
Rab4a	690	RAW264.7	665	EcoRI	atcagaattcatggcgcagaccgccatgtccgaga	666	SalI	gttagtcgaccctgtgggtttagcatttcaggtgtgg
Rab4b	701	RAW264.7	670	EcoRI	atcagaattcatggccgagacctacgacttcctcttc	671	SalI	taaagtcgactttatggaggaccaggacag
Rab5a	702	AtT20	673	EcoRI	cagcgaattcatggctaatcgaggagcaacaagacccaac	674	SalI	acacgtcgacggaatagccaacagtga
Rab5b	707	AtT20	676	EcoRI	ctttgaattcctggctatgactagcag	677	SalI	attagtcgacagcagagctaaaggtgc
Rab5c	241	AtT20	679	XhoI	ggcactcgagaaatggcgggtcgaggaggtgc	680	KpnI	cagaggtaccggtcagagtgaccaagggtgga
Rab6a	708	AtT20	682	EcoRI	aggcgaattcggcggctcctccggctccatcat	683	SalI	agtcgtcgacagctcatgtgaagggaca
Rab6b	710	EST clone GeneBank BC060618	685	EcoRI	tacagaattcatgtccgcagggggagattttggg	686	SalI	gtccgtcgacatggccgactcctctca
Rab7a	1239	Primary mouse fibroblasts	632	XhoI	cgctctcgagcatgacctctaggaagaaagtgttgctg	633	KpnI	cggaggtaccgcaccagccttcccacaggacctt
Rab8a	1048	EST clone GeneBank BC019990	512	EcoRI	agtgaattcatggcgaagacgtacgattatctgttc	514	SalI	catgtcgacaacagcaaaattctaactctctccatc
Rab8b	1049	AtT20	512	EcoRI	agtgaattcatggcgaagacgtacgattatctgttc	513	SalI	gctgtcgacgaagtgcacaggcagacagcaatgctc
Rab9a	313	Melanoma cell line cDNA library	688	XhoI	atcactcgagaaatggcaggaaaatcgtctct	689	KpnI	aagtggtaccccaatttccttgtgggtca
Rab9b	1997	Melan-ink4a melanocyte cell line	691	EcoRI	ccctgaattctgctgcaacatgagtgggaaat	1130	SalI	gtccgtcgacctaacaacaagaagaacttgcttt

Gene	ID #	Source/Cells	Sense #	5´Restriction Site	Sequence	Antisense #	3´Restriction Site	Sequence
Rab10	768	EST clone GeneBank BC056374	694	EcoRI	aagagaattcgagccgctcctcccaatggcg	695	SalI	caatgtcgacgacaaacgctactaagacccactc
Rab11a	773	AtT20	697	EcoRI	tgaggaattcatgggcacccgcgacgacgagta	698	SalI	aatagtcgaccatgctggttgctgaatatggtg
Rab11b	1603	AtT20	700	EcoRI	gccgctcgagaaggaagcgccaggacaatggggac	701	SalI	caacggtaccagggctacagggagaataagag
Rab12	1894	Melanoma cell line cDNA library	703	EcoRI	ccgagcgcgaggaattcgcgtgcatggatc	704	SalI	aatcgtcgacaacaggatttgtgtaggacagcag
Rab14	801	AtT20	709	EcoRI	agcaacccccagtgaattcatggcaactgcaccg	710	SalI	gcaggtcgacgtactgcttccaacagacagagg
Rab15	1390	EST clone GeneBank BC027769	712	EcoRI	gagtcgaattcatggcgaaacagtacgatgtg	713	SalI	ccttgtcgaccgctcttgcctctatctggagttc
Rab18	315	RAW264.7	720	XhoI	gtcggagtctcgagggcgcaggatggacga	721	KpnI	cagacagtggtaccacaacaagttacaactgaggca
Rab20	1402	RAW264.7	726	EcoRI	gcagggaattctcctccgcggctatgcg	727	SalI	ctgaggtcgaccacagtgcaggacgtagtgac
Rab21	1430	EST clone GeneBank BC055042	729	EcoRI	cggggtagcggcgaattcatggctgcggcc	730	SalI	aacacgtcgacgcgcacttccacgttaagac
Rab22a	1604	RAW264.7	854	EcoRI	ctggttctcgagccatggcgctgagggaactt	733	SalI	gatgcggtaccgagagggagaaggaaggaag
Rab23	1457	Melan-ink4a melanocyte cell line	737	XhoI	cttcctgctcgagaaatgttggaggaagatatggaagt	1144	KpnI	atgaggtaccttagggtacactacagctgctgaa
Rab28	1465	AtT20	749	EcoRI	cgggggcagggaattctcctcggacaccat	750	SalI	ttgtagtcgacactctcaatagccagcaaagg
Rab29	1431	EST clone GeneBank BC029056	803	EcoRI	accagaattcatgggcagccgagatcacctgttt	804	SalI	ggacgtcgacaagtcacagacgacacccaagaacaac
Rab31	1991	AtT20	754	EcoRI	ccgacccagaggaattcatggcgatacgggag	755	SalI	agcgggtcgacaccgtcaatgaactgggcac
Rab33a	1433	EST clone GeneBank BC104390	806	EcoRI	ctgtcccgaattcatggcacagcccatcc	807	SalI	gacagtcgacggcttcaacaaggacaggaggctttac
Rab33b	1996	AtT20	809	XhoI	cgggctcgaggtatgacttcggagatggagtcgtcgc	810	KpnI	tagggtaccacagcttacacaccactctgctaa
Rab36	1520	EST clone GeneBank AK038445	763	EcoRI	tactctttacccgaattcttgagtctg	764	SalI	tgtcccaagtcgaccagagataggtggc
Rab38	1241	RAW264.7	634	EcoRI	cgtggaattcatggcgggcgaggactagggcaacag	635	SalI	tcatgtcgacacagctatgtgttatggaatcatgttattt
Rab39b	416	AtT20	772	XhoI	cctgccaggctcgagccatggaggccatctg	773	KpnI	tgaatggtaccaatgaccaagggttgactta
Rab43	1605	AtT20	864	XhoI	actcccctcgagcaatggcgggtcctgg	865	BamHI	aggtggatcccaagggtaggatgtcagca
LC3	2198	Hepa1-6	1471	EcoRI	gccgaattcgaagtgatcgccggagtc	1472	SalI	gcccgtcgacatgtgggtgcctacgttctc
OSBP		Hepa1-6	1172	EcoRI	GCGCCGAATTCtcgggctcggctagagagggctg	1173	SalI	tgatgtcgacagctacttcacagccttggccttg

Supplementary Table 3

List of genes tested in the siRNA screen and Plasmodium berghei infection rates. Table is organized from the highest to the lowest infection rate value (total number of parasites divided by the number of nuclei per well). * Mean Infection rate of triplicate wells relative to median infection rate of entire plate. ** Mean Z-Score of triplicate wells relative to median Z-score of entire plate.

Well number	Well name	Gene Symbol	Mean Infection Rate*	Mean Z-Score**
16	C07	Rab1a	2,261	4,256
13	C04	Ggps1	1,649	2,099
20	C11	Rab3a	1,647	2,610
56	G07	Rab28	1,619	2,493
33	E04	Rab8a	1,569	2,189
15	C06	RabGGTB	1,475	1,887
50	F11	Rab23	1,415	1,287
30	D11	Rab6b	1,394	1,475
12	C03	Chml	1,393	1,339
11	C02	Praf2	1,392	1,341
38	E09	Rab11a	1,332	-1,078
42	F03	Rab14	1,315	1,095
24	D05	Rab4a	1,253	0,926
57	G08	Rab7L1	1,250	0,978
5	B06	siControl	1,220	0,839
26	D07	Rab5a	1,212	0,661
2	B03	siControl	1,207	0,886
21	D02	Rab3b	1,179	0,976
14	C05	Fdps	1,151	0,507
37	E08	Rab10	1,142	0,611
29	D10	Rab6	1,118	0,663
49	F10	Rab22a	1,113	0,588
52	G03	Rab25	1,107	0,617
43	F04	Rab15	1,087	0,512
23	D04	Rab3d	1,081	0,562
17	C08	Rab1b	1,070	0,336
7	B08	siControl	1,069	0,190
48	F09	Rab21	1,068	0,347
25	D06	Rab4b	1,059	0,234
36	E07	Rab9b	1,039	0,262

Well number	Well name	Gene Symbol	Mean Infection Rate*	Mean Z-Score**
35	E06	Rab9	1,035	0,222
39	E10	Rab11b	1,022	0,174
53	G04	Rab26	0,999	0,338
3	B04	siControl	0,973	-0,019
32	E03	Rab7L1	0,963	-0,067
41	F02	Rab13	0,959	-0,087
47	F08	Rab20	0,944	0,211
55	G06	Rab27b	0,944	-0,080
10	B11	siControl	0,930	-0,131
45	F06	Rab18	0,916	-0,147
6	B07	siControl	0,916	-0,073
54	G05	Rab27a	0,912	-0,072
40	E11	Rab12	0,910	-0,075
51	G02	Rab24	0,906	-0,214
8	B09	siControl	0,896	-0,435
9	B10	siControl	0,894	-0,142
28	D09	Rab5c	0,890	-0,077
59	G10	Rab31	0,887	-0,238
34	E05	Rab8b	0,884	-0,320
31	E02	Rab7	0,876	-0,156
4	B05	siControl	0,849	-0,431
60	G11	Rab32	0,844	-0,268
46	F07	Rab19	0,792	-0,587
58	G09	Rab30	0,791	-0,575
27	D08	Rab5b	0,781	-0,697
44	F05	Rab17	0,775	-0,650
1	B02	siControl	0,754	-0,684
18	C09	Rab2	0,753	-0,755
19	C10	Rab2b	0,732	-0,977
22	D03	Rab3c	0,728	-0,817