

THE LIVER ENDOPLASMIC RETICULUM IN MALARIA LIVER INFECTION

A FUNCTIONAL STUDY OF THE UNFOLDED PROTEIN RESPONSE

PATRÍCIA IRENE DA SILVA INÁCIO

DISSERTATION PRESENTED TO INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA
UNIVERSIDADE NOVA DE LISBOA TO OBTAIN THE PhD IN BIOLOGY,
SPECIALIZATION IN CELLULAR BIOLOGY, 2013

The work presented in this thesis was developed during my PhD research project. The work was performed under the supervision of Doctor Maria Manuel Mota, Instituto de Medicina Molecular, Faculty of Medicine, Universidade de Lisboa, Portugal and co-supervised by Doctor Adriano Henriques, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa.

This thesis has been supported by the grant SFRH/BD/33221/2007 from Fundação para a Ciência e a Tecnologia, Lisboa, Portugal and by Bolsas C&T – I&D Concurso 2012 from Fundação Luso-Americana.

TABLE OF CONTENTS

11	ACKNOWLEDGMENTS
15	ABBREVIATIONS
21	SUMMARY
23	SUMÁRIO
27	INTRODUCTION CHAPTER ONE
27	1 MALARIA
27	1.1 Major achievements and future challenges
29	1.2 <i>Plasmodium</i> life cycle
32	1.3 The mammalian host
32	1.3.1 The erythrocytic stage of <i>Plasmodium</i> infection
34	1.3.2 The pre-erythrocytic stage of <i>Plasmodium</i> infection
34	1.3.2.1 The skin stage
34	1.3.2.2 The liver stage
34	1.3.2.2.1 Arrest in the liver
35	1.3.2.2.2 Hepatocyte invasion
38	1.3.2.2.3 Parasite development in hepatocytes
39	1.3.2.2.4 Exit the liver to the blood
42	1.4 Models for <i>Plasmodium</i> liver stage infection
43	2 THE LIVER
43	2.1 The Endoplasmic Reticulum
44	2.2 The mammalian UPR
45	2.2.1 The IRE1 α -XBP1 pathway
48	2.2.2 The PERK-eIF2 α pathway
52	2.2.3 The ATF6 pathway

52	2.2.4 CREBH – a liver specific ER stress transcription factor
54	2.3 ER, Metabolism and UPR
54	2.3.1 UPR in Lipid Metabolism
57	2.3.2 UPR in Glucose Metabolism
59	2.3.3 UPR Crosstalk with Iron Metabolism
61	2.4 UPR and Inflammation
64	3 STUDY OUTLINE
69	RESULTS CHAPTER TWO
75	4.1 The role of UPR activation in <i>Plasmodium</i> liver infection
94	4.2 Impact of discrete UPR pathways on <i>Plasmodium</i> liver infection
94	4.2.1 The XBP1 pathway
100	4.2.2 The CREBH pathway
111	DISCUSSION CHAPTER THREE
119	CONCLUSIONS AND FUTURE CHALLENGES CHAPTER FOUR
123	MATERIALS AND METHODS CHAPTER FIVE
131	REFERENCES

ACKNOWLEDGMENTS

The completion of my PhD wouldn't have been possible without the many people who I have had the pleasure of working with. First of all, I must thank the Gulbenkian PhD Programme where I started. Obrigada pela incrível oportunidade de poder ter feito parte deste programa, de ter conhecido e ter trabalhado com todos os meus colegas dos três programas doutorais da Gulbenkian.

Um sincero obrigada à Maria! Obrigada por me teres aceite no teu laboratório, pela liberdade e oportunidade que me deste para poder pensar e aprender.

Obrigada ao Doutor Adriano Henriques pelos comentários e entusiasmo com o meu trabalho.

To Gunnar, thank you for everything!! For all your support, for the discussions about the work (and beyond!).

Ao Bruno Silva-Santos, muito obrigada pelo entusiasmo e comentários acerca do trabalho.

Special thanks to David Fidock and Brie Falkard, for receiving me in New York and for all your support and enthusiasm.

To Vanessa, Nagarjuna Nagaraj and Matthias Mann I thank for allowing me to use the proteomics screen.

To Koos Rooijers, thank you for all your precious help.

Ao Nuno, obrigada pela ajuda com todas as figuras da tese e por estares sempre disponível a ajudar.

I thank all the members of Mota's lab, past and present, with whom I learned so, so much - in particular to Vanessa, obrigada por me teres ensinado e acompanhado quando cheguei ao laboratório; Fernanda (obrigada!) and Ana Parreira, Ana Roberto, Céline, Liliana, Daniel, Miguel, Ghislain, Silvia, Peter, Claudia, Ksenija, Maurice, Kirsten, António, Catarina, Margarida, Audrey, Joana Carrelha, Joana Dias, Rosangela e Maria Rebelo; Iset (thank you for all your help!); Inês (ou melhor, Maria Alice) e Meireles, obrigada pela boa disposição e pela ajuda, sempre que precisei!

Obrigada Rita Neres e Eva (Pamplona's group).

To all the members of UPAMOL, Jorge, Patrícia, Ana, Natalia and Neuza, thank you all for having me in the lab, and for all your precious help!

To all the members of Figueiredo's lab, thank you for being such a nice group to share a space with and, in particular to Ana Pena (obrigada por tudo!) e Fabien (merci!).

My special thanks to Schmolka (danke, meine süße freundin deutsch), Eli, Penérrima, Filipa, Carina, Mariana and Pamplona for being there!

Ao Charlie Brown, por seres sempre o sítio onde quero chegar e permanecer.

E, à minha família maravilhosa, em especial, aos meus pais, ao meu irmão e ao meu avô, pelo apoio incondicional. Dedico todo o meu trabalho à minha avó Irene!

ABBREVIATIONS

Acc2 acetyl-CoA carboxylase 2

ACTs artemisinin-based combination therapies

Acl ATP citrate lyase

AD activation domain

AMA-1 apical membrane antigen 1

ASK1 apoptosis signal-regulating kinase 1

ATF4 activating transcription factor 4

ATF6 activating transcription factor 6

ATP adenosine triphosphate

BiP binding immunoglobulin protein

CEBPa CCAAT enhancer binding protein α

CHOP C/EBP homologous protein

CREBH cAMP responsive element-binding protein, hepatocyte specific

CReP constitutive repressor of eIF2 α phosphorylation

CRP C-reactive protein

CRTC2 CREB-regulated transcription coactivator 2

CSP circumsporozoite protein

DBD DNA-binding domain

DBL Duffy binding-like

DDT dichlorodiphenyltrichloroethane

DE differentially expressed

Dgat2 diacyl glycerol acyl transferase 2

EDEM1 ER degradation enhancer, mannosidase alpha-like 1

eIF2 α eukaryotic translation factor-2 α

ER endoplasmic reticulum

ERAD ER-associated degradation

ERO1 ER oxidoreductin 1

ERSE-I | ERSE-II ER stress-response elements I and II

Fas fatty acid synthase

GO gene ontology

G6Pase glucose-6-phosphatase

GRP94 glucose-regulated protein 94

HSPGs heparan sulphate proteoglycans

HERPUD1 homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1

HGF hepatocyte growth factor

HRD1 ubiquitin protein ligase

IL-4 interleukin-4

IL-6 interleukin 6

IL-8 interleukin 8

iRBCs infected red blood cells

IRE1 α inositol requiring protein 1 α

IRS indoor residual spraying

ISR integrated stress response

ITNs insecticide-treated mosquito nets

iv intravenously injection

JNK stress-induced Jun N-terminal kinase

LDLR low-density lipoprotein receptor

L-FABP liver-fatty acid binding protein

LLINs long-lasting insecticidal nets

MCP-1 monocyte chemotactic protein-1

MSP1 merozoite surface protein 1

MSPs merozoite surface proteins

NF- κ B nuclear factor-kappa B

NK natural killer cells

NKT natural killer T cells

NMR nuclear magnetic resonance

OASIS old astrocyte specifically induced substance

ORFs open reading frames

PC phosphatidylcholine

PDI protein disulfide isomerase

PERK protein kinase RNA (PKR)-like ER kinase

PfRh *Plasmodium falciparum* reticulocyte binding protein homolog

PGC-1 α peroxisome proliferator-activated receptor-gamma coactivator α

pi post-infection

PKC ζ protein kinase C ζ

PPAR α peroxisome proliferator-activated receptor α

PVM parasitophorous vacuole membrane

qRT-PCR quantitative RT-PCR

RDTs rapid diagnostic tests

RIDD regulated IRE1-dependent decay

RIP regulated intramembrane proteolysis

RNAse endoribonuclease

ROS reactive oxygen species

S1P site 1 protease

S2P site 2 protease

SAP serum amyloid P-component

SCAP SREBP cleavage-activating protein

Scd-1 stearoyl-CoA desaturase-1

SPECT sporozoite protein essential for cell traversal

SR-BI scavenger receptor B type I

SREBPs sterol regulatory element-binding-proteins

TLRs toll like receptors

TM tunicamycin

TNF- α tumor necrosis factor α

TRAP thrombospondin-related adhesive protein

TRB3 tribbles homolog 3

UIS3/4 upregulated in infective sporozoites

uORFs upstream open reading frames

UPR Unfolded Protein Response

UPRE UPR element

WHO World Health Organization

XPB1 X-box binding protein 1

XPB1s XPB1 spliced

XPB1u XPB1 unspliced

SUMMARY

Malaria, a disease caused by *Plasmodium*, represents a major health problem with a still disconcertingly high mortality rate (655 000 malaria deaths were estimated by the World Health Organization in 2012), mainly in Africa [1]. After a bite by an infected *Anopheles* mosquito occurs, *Plasmodium* sporozoites reach their target organ, the liver, within minutes. After traversing several hepatocytes, the parasite invades a final one and establishes a parasitophorous vacuole, where it replicates exponentially generating thousands of infective merozoites, the red blood cell infectious forms that are released in the blood stream. The liver stage is the first obligatory phase of malaria infection and, although no symptoms are associated with it, it is absolutely crucial to the establishment of a successful infection.

Inside hepatocytes the parasite establishes a replicative niche that was shown to impact hepatocytes metabolic transcriptional programmes [58] such as lipid, glucose and aminoacids metabolism, thus suggesting that *Plasmodium* development is intrinsically connected to hepatocytes metabolic status. In highly metabolically active cells, such as hepatocytes, the endoplasmic reticulum (ER) is essential to maintain cellular homeostasis. In fact, the ER is increasingly being recognized as a metabolic organelle due to its role not only as the primary organelle of cellular anabolic metabolism, but also by being a signal-integrating platform of the metabolic status of the cell. We hypothesized that *Plasmodium* development within hepatocytes impacts ER functions and modulates the unfolded protein response (UPR) signaling pathways. To address this question we analysed a semi-quantitative proteomic screen and a transcriptome study between sporozoite infected versus uninfected cells, in order to determine the impact of infection on the host ER. We found a significant modulation of ER proteins with infection, in agreement with a clear ER stress signature and concomitant UPR activation. To understand the role of the UPR in the infection we induced its activation in the liver prior to sporozoite delivery. Our data clearly showed that UPR activation greatly enhances *Plasmodium* liver infection. We explored this

phenotype and discovered that the mechanism leading to infection increase is multifactorial. We found that, in the liver, sporozoite infection induces a potent type I interferon response and that UPR activation abolishes it. Beyond its role in innate immunity we found that UPR activation increases infection through regulation of different metabolic pathways. Specifically, we found that the UPR branch mediated by XBP1 active form, XBP1s, is induced by *Plasmodium* infection together with the liver specific branch mediated by CREBH. Both branches contributed to *Plasmodium* infection through their regulatory actions on different metabolic pathways: XBP1 possibly by enhancing *de novo* lipogenesis and CREBH by regulating iron availability via hepcidin.

These findings suggest that ER stress is a central feature of malaria liver infection and that activation of the unfolded protein response contributes to the parasite infection, therefore establishing the ER as a new mediator in liver stage malaria.

SUMÁRIO

A malária, uma doença causada pelo parasita do género *Plasmodium*, representa um grave problema de saúde pública, principalmente em África, onde ainda se verifica uma elevada taxa de mortalidade (a Organização Mundial de Saúde estimou 655 000 óbitos associados à malária em 2012) [1]. O parasita *Plasmodium* é transmitido, sob a forma de esporozoítos, com a picada do mosquito *Anopheles*, atingindo o fígado em minutos através da circulação sanguínea. Uma vez no fígado, o parasita atravessa vários hepatócitos antes de estabelecer a infecção num hepatócito, através da formação de um vacúolo parasitóforo, onde se desenvolve e replica em dezenas de milhares de merozoítos (os parasitas responsáveis pela infecção dos eritrócitos, uma vez libertados na corrente sanguínea). A fase hepática é uma etapa obrigatória da infecção do hospedeiro vertebrado e, apesar de não estar associada a sintomas clínicos, é uma fase indispensável para a progressão da doença.

No interior da célula infectada, cada parasita estabelece um nicho onde se desenvolve e replica, e foi demonstrado que a infecção altera o perfil de expressão genética associada ao metabolismo dos hepatócitos [58]. Especificamente, a expressão de genes associados ao metabolismo de lípidos, glucose e aminoácidos o que sugere que a infecção pelo parasita está intimamente associada ao metabolismo da célula hospedeira. Em células metabolicamente muito activas, tais como os hepatócitos, o retículo endoplasmático (RE) é essencial na manutenção da homeostasia celular. O RE é, aliás, considerado um organelo metabólico, por ser o principal organelo responsável pelo anabolismo celular, mas também por responder e regular o metabolismo celular através de uma complexa via denominada *Unfolded Protein Response* (UPR). Sendo assim, neste estudo colocámos a hipótese de que a infecção hepática poderá ter um impacto nas funções do RE, resultando na modelação das vias integradas na UPR. De forma a testar esta hipótese, procedemos à análise de dois estudos, um *screen* semi-quantitativo de proteínas e um estudo de *microarrays* entre células infectadas e não infectadas, para determinar o impacto da infecção no RE dos hepatócitos. Esta análise permitiu identificar

alterações significativas de proteínas do RE, características de uma resposta de stress do RE e activação da *UPR*, em células infectadas. Para avaliarmos o impacto da *UPR* na infecção pelo parasita, procedemos à sua activação no fígado imediatamente antes da infecção. Esta análise demonstrou que a activação da *UPR* aumenta drasticamente a infecção. Posteriormente, um estudo mais detalhado revelou que a causa do aumento na infecção é multifactorial. No fígado, os esporozoítos induzem uma resposta de interferão tipo I que é significativamente reduzida com a activação da *UPR*. Paralelamente, a activação da *UPR* aumenta a infecção ao regular diferentes vias de metabolismo nos hepatócitos. Especificamente, as vias da *UPR* regulada pela forma activa de XBP1, XBP1s, e a via específica do fígado mediada pelo CREBH são induzidas com a infecção. Ambas as vias contribuíram para a infecção hepática ao regular diferentes aspectos do metabolismo hepático: a via regulada pelo XBP1, possivelmente, através da indução da *de novo* lipogénese e o CREBH ao regular o metabolismo do ferro, via a hormona hepcidina.

Este estudo demonstra que o stress do RE é uma condição característica da infecção hepática e que a consequente activação da *UPR* contribui para a viabilidade do parasita na célula hepática hospedeira.

INTRODUCTION

CHAPTER ONE

1 MALARIA

1.1 Major achievements and future challenges

Malaria still represents a major health problem, particularly in Africa. In 2010, 216 million new cases of malaria and 655 000 malaria deaths were estimated by the World Health Organization (WHO), 81% and 91% (with up to 86% deaths in children under 5 years old) in the Sub-Saharan region, respectively [1]. Although these numbers represent a major achievement (malaria mortality rates have fallen by more than 25% globally since 2000 and by 33% in the African Region), malaria remains one of the most prevalent infectious diseases worldwide with a mortality rate still disconcertingly high (FIGURE 1). The real numbers may be even higher, as a recent study using a systematic analysis indicated that global malaria toll exceeded 1.2 million in 2010 [2].

The past decade witnessed unprecedented efforts in malaria control. Access to insecticide-treated mosquito nets (ITNs) has increased by 50% since the year of 2000. The number of rapid diagnostic tests (RDTs) and artemisinin-based combination therapies (ACTs) has also expanded significantly. Currently, a number of strategies and products represent our tool box in fighting malaria. Rapid diagnostic tests (RDTs) that can be used in the field have been positively reinforced by the latest WHO directives. In countries where health systems may be fragile and a population fraction might not easily access to it, a rapid and reliable diagnostic is critical to confirm every suspicion of malaria before treatment. With the exception of the regions where chloroquine remains fully effective, artemisinin-based combination therapies (ACTs) are recommended to treat uncomplicated *P. falciparum* malaria. Monotherapies as treatment of malaria are not to be followed in order to prevent resistance and chemoprophylaxis, as a preventive measure, is applied to pregnant women and children in specific areas of high infection risk. For decades, the scientific community has pursued a vaccine against malaria. At the moment, the vaccine candidate RTS,S is on phase 3 of clinical trials although the efficacy does

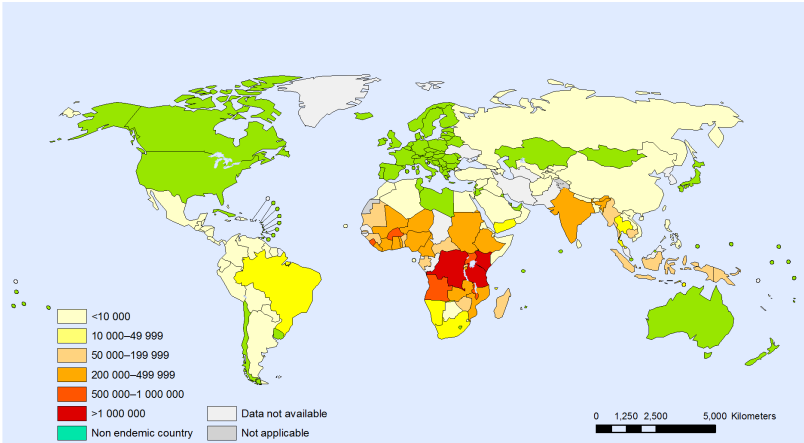


FIGURE 1
Number of malaria reported confirmed cases in 2010 by World Health Organization (WHO).

not go beyond 30% [3]. Moreover, vaccine's efficacy declined quickly over time and with increasing malaria exposure, as recently reported in a 4 years follow-up study where the efficacy registered was only 16.8% [4]. Insecticide-based vector-control interventions contributed greatly for the global progress in fighting malaria, mainly the long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) initiatives. In LLINs the only class of insecticides used is the pyrethroids. Four classes of insecticides, including DTT (dichlorodiphenyltrichloroethane) are used in IRS.

Despite all these achievements, crucial aspects of *Plasmodium* biology remain elusive: *Plasmodium* repeated infection ultimately leads to protective immunity, however our knowledge of naturally acquired immunity is remarkably scarce. Although malaria symptoms arise only once the parasite infects red blood cells, the liver stage is an essential and obligatory step that still remains largely unknown. Importantly, besides the more basic aspects of malaria research, practical issues are also arising. Recent evidence that resistance to artemisinin derivatives, currently the first line treatment to combat malaria, is developing in Southeast Asia denotes the urgency to, not only develop new antimalarial compounds, as well as to understand the mechanisms of resistance. Additionally, while our resources are mainly directed to *Plasmodium falciparum* malaria, the agent responsible for the most severe forms of disease nowadays, *Plasmodium vivax* is a more widespread parasite and increasingly cases of infection with *Plasmodium knowlesi* have been reported, endangering 40% of the world's population. With a more complex biology, it is believed that *Plasmodium vivax* will be more difficult to control and we do not have enough and adequate tools to fight it.

In the last decade, malaria research in all its fronts produced the knowledge and tools responsible for the encouraging results in malaria control. But these gains are still fragile. Strengthening can only be achieved by the joint work of basic and clinical research.

1.2 *Plasmodium* life cycle

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*. The malaria parasite is the most important member of the Apicomplexa phylum, which includes other parasites of human and also veterinary importance as *Toxoplasma*, *Theileria*, *Eimeria*, *Babesia*, and *Cryptosporidium*.

Plasmodium primary natural transmission vector are female *Anopheles* mosquitoes (however, *Plasmodium* can also be transmitted by pregnant women to their unborn child). There are approximately 5000 *Plasmodium* species which infect animals ranging from humans and other mammals to birds and lizards. From those, five can cause malaria in humans – *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*

and *Plasmodium knowlesi*. *Plasmodium falciparum* causes the majority of infections in Africa and is responsible for the most severe forms of disease and mortality. *P. vivax* and *P. ovale* form dormant forms in the liver (hypnozoites) that, once reactivated, can cause clinical relapses, i.e., the re-appearance of clinical symptoms of a disease after its cessation [5].

Parasites of the genus *Plasmodium* are obligate intracellular organisms with a complex life cycle, switching between a mosquito vector and a mammalian host (FIGURE 2). The mammalian life cycle begins when an infected female *Anopheles* mosquito, during its blood meal, deposits *Plasmodium* parasites (named at this stage, sporozoites) under the skin of the mammalian host. Once in the bloodstream, these extracellular forms rapidly migrate to the liver and actively invade hepatocytes. Each sporozoite differentiates and divides into thousands of liver merozoites that are released into the bloodstream, between 2 to 16 days later, depending on the species. Each merozoite invades an erythrocyte, where after a replication cycle the infected erythrocyte (schizont) ruptures and releases newly formed merozoites which are ready to infect other erythrocytes. This begins the asexual blood-stage life cycle of *Plasmodium* parasites. In the blood of the mammalian host another parasite cycle also occurs. While the asexual *Plasmodium* life cycle goes on, some merozoites cease asexual replication and switch to sexual development with the formation of male and female gamete precursors (gametocytes). Although the sexual stages exist in a reduced number, when compared to the asexual one, it is of crucial importance to assure malaria transmission. What triggers gametocytogenesis is still to be determined. During a mosquito blood meal, occurs the uptake of female and male gametocytes. Once in the mosquito midgut, gametocytes undergo fertilization and maturation leading to an infective ookinete. Similarly to sporozoites, ookinetes are motile and cross the midgut epithelial cell wall. Between this and the basal lamina of the midgut, the ookinete develops into an oocyst. Once matured, the oocyst burst and releases the thousands of sporozoites into the hemocoel (body cavity) that will then invade the salivary glands. Here the sporozoites are ready to be injected in a new host, thus beginning a new cycle (reviewed in [6]).

In the mammalian host, malaria associated symptoms only occur during the blood stage of infection. Thus, the disease progresses from an asymptomatic, silent stage – pre-erythrocytic – to a symptomatic one – the erythrocytic stage. For the purpose of this thesis, I will first briefly present the erythrocytic stage to then focus on our current knowledge of the pre-erythrocytic stages of infection, with main focus on the liver stage.

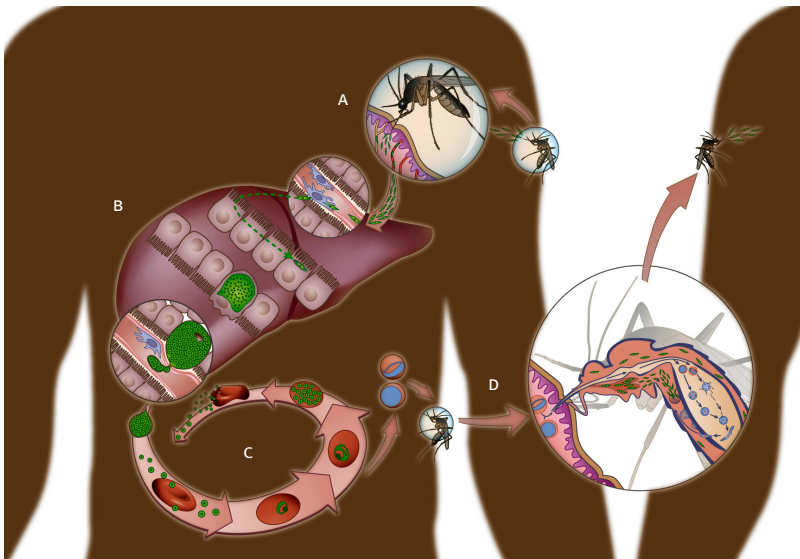


FIGURE 2

***Plasmodium falciparum* life cycle.**

During a blood meal, female Anopheles mosquitoes inject the sporozoite form of the parasite into the skin of a human host (A) The sporozoites travel to the liver via the bloodstream and, once inside hepatocytes, replicate into thousands of merozoites (B) Merozoites are released in the blood where an asexual cycle occurs with invasion and replication within red blood cells, followed by the production of female and male gametocytes (C) The gametocytes are transmitted back to a mosquito during a blood meal, where they fuse to form oocysts that divide into new sporozoites. These migrate to the salivary glands, starting a new infection cycle with the next blood meal (D).

1.3 The mammalian host

1.3.1 The erythrocytic stage of *Plasmodium* infection

Once released into the bloodstream hepatic merozoites recognize, attach and invade erythrocytes, a rapid process that occurs within 1 minute [7]. The invasion of erythrocytes occurs in four distinct phases – adhesion, reorientation, tight-junction formation and invasion. Due to the lack of cilia or flagella, merozoites when released into the bloodstream rely on passive diffusion to access the red blood cell. The adhesion process is suggested to be mediated by merozoite surface proteins (MSPs), however definitive proof is still lacking. On the contrary, the role of two protein families, the DBL (Duffy binding-like) and PfRh (*P. falciparum* reticulocyte binding protein homolog) family is well established [7]. Recognition and adhesion is crucial to ensure the binding to the correct cell. Once attached to the red blood cell, merozoite reorients itself juxtaposing its apical end (with the rhoptry and microneme organelles) towards the erythrocyte membrane. Once apical attachment is established, several proteins stored in these organelles bind to specific receptors on the erythrocyte creating a tight-junction [8]. During invasion, the tight junction moves from the apical end to the posterior pole of the merozoite powered by the actin-myosin motor of the parasite [9]. As the parasite invades it creates a parasitophorous vacuole (for a detailed review see [10]).

While parasite proteins as DBL and PfRh are important for erythrocyte invasion they are not essential. *P. falciparum* not only has multiple ligand-receptor interactions, named invasion pathways, as it is able to switch between them. This capacity represents a strategy that allows the parasite to evade host receptor polymorphisms and immune system responses [11]. However, a recent study showed that *P. falciparum* relies on a single receptor, known as ‘basigin’, on the surface of red blood cells to invade the cell. Basigin is a receptor for PfRh5, a parasite ligand that is essential for blood stage growth [12]. This cross-strain dependency on a single extracellular receptor-ligand has recently been shown to offer a potential target for a new anti-malarial therapy, since PfRH5 generated an immune response in animal models capable of neutralising many of the strains of the *P. falciparum* parasite tested [13].

Inside erythrocytes, the merozoites develop for 48 hours beginning with the ring stage (0-24 hours), followed by DNA replication and growth during the trophozoite stage (24-36 hours) and, finally, the schizont stage (36-48 hours) culminating with 16 to 32 new merozoites that are released during

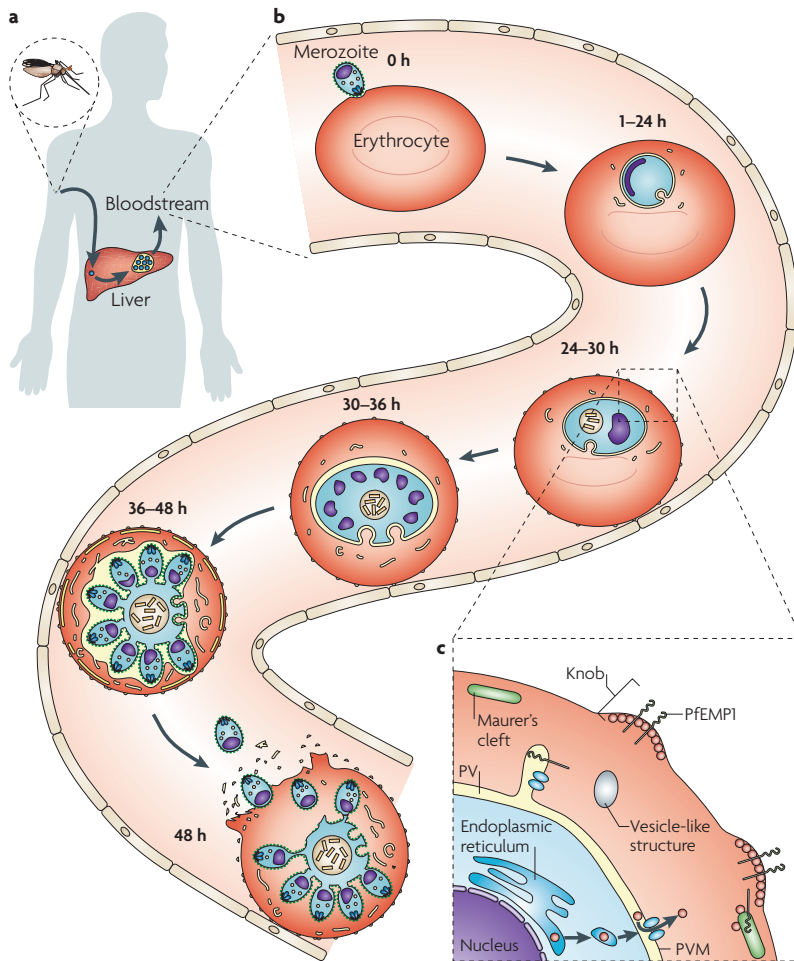


FIGURE 3
***Plasmodium falciparum* blood stage development.**

Plasmodium falciparum merozoites attach to and invade erythrocytes, with the formation of a vacuole where the parasite develops through the ring (0–24 hours), trophozoite (24–36 hours) and schizont stages (40–48 hours). A small percentage of the parasites undergo differentiation into gametocytes (not shown), which can be taken up by a mosquito during a blood meal and start a new infection cycle. In order to survive, the parasite remodels its host cell by exporting hundreds of parasite proteins into the erythrocyte. The export of one parasite protein is shown, starting with its secretion from the endoplasmic reticulum and into the parasitophorous vacuole (PV).

An export machinery is responsible for the transport of proteins pass beyond the parasitophorous vacuolar membrane (PVM). Exported proteins, as PfEMP1, are part of the knobs and the Maurer's clefts which are responsible for infected erythrocytes cytoadherence (Adapted from Goldberg and Cowman, 2010).

red blood cell egress (FIGURE 3) to start a new replication cycle.

The egress from the infected erythrocyte involves rupture of its parasitophorous vacuole and host cell membrane. Egress is mediated by an increase in intracellular pressure together with a destabilization of the infected cell cytoskeleton via biochemical changes. This mechanical/biochemical forces induce the erythrocyte explosive rupture and merozoite release [14]. Although the cause for the increase in pressure and cytoskeleton disruption is not clear it appears to occur in a two-step process [15]. Accordingly, the internal vacuolar membrane is disrupted prior to the erythrocytic one in a protease depend event [16].

1.3.2 The pre-erythrocytic stage of *Plasmodium* infection

1.3.2.1 The skin stage

During the bite of a female *Anopheles* mosquito, the insect probes for a blood vessel in the skin of the vertebrate host. It was believe that the sporozoites located at the mosquito salivary glands were directly delivered into the skin blood vessels during the mosquito blood meal. This concept was challenged when a series of studies showed that sporozoites are actually deposited in the dermis ([17-19]). Sporozoites are injected at an average between 100 to 200, during a mosquito bite ([20, 21]). After injection into the skin, sporozoites migrate through the dermis in order to reach a blood vessel and enter the circulatory system to reach their target organ – the liver [17, 19]. Although sporozoites, once start migrating, are able to invade a blood vessel within a minute, a significant proportion of sporozoites remain in the dermis (approx. 50%) [17]. In fact, sporozoites were found in the dermis up to 7 hours after injection. Of those that leave the bite area within 1 hour, approximately 70% reach blood vessels, while the remaining 30% invade lymphatic vessels. Lymph sporozoites cease in the proximal lymph node, where the majority is degraded by dendritic cells [17]. Thus, within a short period of time sporozoites have to be able to migrate to a blood vessel in order to establish a successful liver infection.

The termed “skin stage” has been suggested as a new pre-erythrocytic stage in malaria infection and possibly the first stage to boost a host immune response against sporozoites [22].

1.3.2.2 The liver stage

1.3.2.2.1 Arrest in the liver

As soon sporozoites reach the circulatory system, they are found in hepatocytes within 2 minutes of intravenous injection into rats, despite contacting

and traversing many cell types as they migrate from the skin to the liver [23]. The specificity of the target cells suggests a strong and exact interaction between parasite surface proteins and hepatocyte molecules. The molecules involved in sporozoite arrest in the liver have been extensively studied, from host and parasite side.

In the host, the heparan sulphate proteoglycans (HSPGs) at the surface of liver cells have been shown to be the trigger for the preferential attachment of *Plasmodium* receptors [24, 25]. Liver sinusoids are lined by fenestrated endothelial cells, have resident Kupffer cells and are separated from hepatocytes through the space of Disse. Here, we can find the stellate cells, which are responsible for the continuous production of the extracellular matrix [26]. It has been suggested that the initial arrest of *Plasmodium* sporozoites in the liver sinusoids occurs by binding to the proteoglycans produced by stellate cells (FIGURE 4A). The proteoglycans traverse endothelial cells fenestrations encountering the sporozoites in the sinusoidal lumen. Moreover, stellate cells synthesize eight times more proteoglycans than hepatocytes, with twice the level of sulphate [27]. Once arrested in the liver sinusoid, proteoglycans at the surface of Kupffer cells mediate sporozoite entry into hepatocytes [28].

In the parasite, two proteins, the circumsporozoite protein (CSP) and the thrombospondin-related anonymous protein (TRAP) recognize and bind to the HSPGs in the basolateral membrane of hepatocytes in the space of Disse ([24, 29]).

HSPGs are not exclusive to liver cells, however, sporozoites react only to these proteins in the liver. Studies from different groups suggest that the HSPGs high levels of sulphation in liver cells [30] are the trigger for sporozoite arrest in the liver and invasion of hepatocytes [31, 32].

1.3.2.2.2 Hepatocyte invasion

Once sequestered in the liver sinusoids, sporozoites must reach and invade the hepatocytes. However, in the route to hepatocytes lies the sinusoidal cell layer composed of endothelial and Kupffer cells. According to some studies, sporozoites reach hepatocytes by invading Kupffer cells with the formation of a non-acidic vacuole [33]. However, transgenic parasites with a defect in the traversing machinery (SPECT and SPECT2 mutants) have reduced *in vivo* infectivity, suggesting that the crossing towards hepatocytes is dependent on cell membrane disruption. Additionally, Kupffer cell depletion restored the *in vivo* infectivity to the SPECT mutant sporozoites which supports the role of Kupffer cells in hepatocyte invasion [34, 35]. Why Kupffer cells do not recognize and remove, by phagocytosis, the traversing sporo-

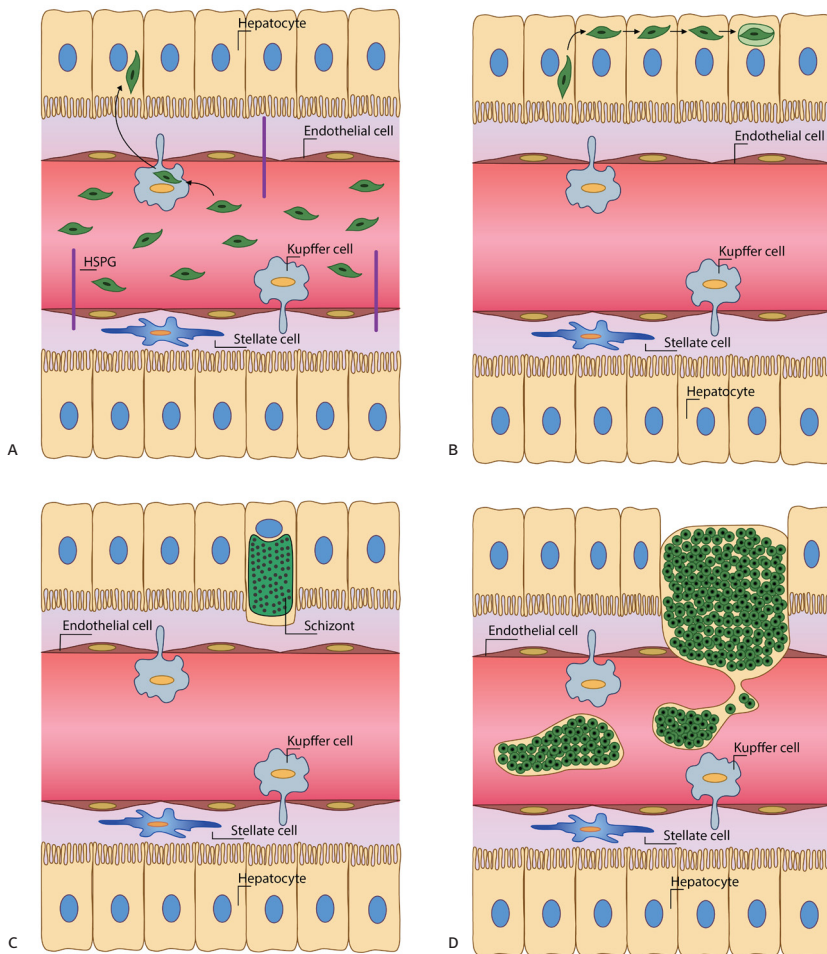


FIGURE 4

***Plasmodium* liver stage.**

Plasmodium sporozoites arrest in the liver – sporozoites (green) present in the liver sinusoids arrest in the liver by binding to heparan sulphate proteoglycans (HSPGs) from stellate cells and hepatocytes; they enter hepatocytes by traversing the sinusoidal layer, possibly through the Kupffer cells (A) *Plasmodium* replication within hepatocytes – once the sporozoite enters an hepatocyte, it traverses several before invasion of a final one where it establishes a parasitophorous vacuole (B); each sporozoite replicates and develops inside its vacuole forming the schizont that contains thousands of merozoites (C) *Plasmodium* release into the bloodstream – with merozoites fully developed, its

release into the bloodstream occurs via merosomes (vesicles full of merozoites), which bud off from the infected hepatocyte into the sinusoidal lumen (D) (Adapted from Prudencio et al., 2006).

zoites is still not known.

After crossing the space of Disse, sporozoites traverse several hepatocytes before invading a final one where it will establish its parasitophorous vacuole (FIGURE 4B). The traversal of hepatocytes is achieved by breaching the cell membrane [36]. The requirement of cell traversal by *Plasmodium* to establish a successful liver infection has been challenged by studies with *Plasmodium* mutants that lost the ability to migrate. Some studies defend that migration through cells leads to sporozoite regulated exocytosis to expose membrane proteins, TRAP, activating sporozoites for hepatocyte invasion [37], and induces the secretion of the hepatocyte growth factor (HGF). Locally increased HGF levels are thought to sustain the survival of infected hepatocytes via HGF/MET signalling and the activation of anti-apoptotic pathways [38]. However, deletion of the *spect* gene completely abrogates the sporozoite capacity to traverse cells, while maintaining their infectivity *in vitro* in both hepatoma cells and primary hepatocytes [35]. The same phenotype was maintained with the deletion of other traversal-related genes [34, 39]. Thus, while migration through cells is essential for the parasite to be able to reach the liver parenchyma, it is dispensable to infect liver cells after passing the natural barriers.

Plasmodium sporozoites invade the final hepatocyte, after migration ceased, to establish their replication niche within a parasitophorous vacuole. Hepatocyte invasion is thus an active process that involves parasite and host molecules. From the parasite side, the microneme proteins CSP, TRAP and AMA-1 (apical membrane antigen 1) have been implicated in hepatocyte invasion ([24, 29, 40]). The role of two proteins specifically produced in liver-sporozoites, Pb36 and P36p, in hepatocyte invasion is still a matter of debate. While the first study with knockout parasites for both proteins nearly abolished sporozoite infectivity [41], recently it has been proposed that although the infectivity does not change, the formation of the parasitophorous vacuole is largely compromised [42, 43].

From the host side, the already mentioned HSPGs have an important role in binding and internalization of sporozoites, due to their interaction with CSP and TRAP parasite proteins. Serum glycoprotein fetuin A by interacting with TRAP extracellular region enhances hepatocyte invasion by *Plasmodium* [44]. Two other proteins, the tetraspanin CD81 and SR-BI (scavenger receptor B type I), both membrane proteins expressed at the surface of hepatocytes, have been shown to play a role in hepatocyte infection. CD81 deletion from hepatocytes, *in vitro* and *in vivo*, inhibits parasite invasion [45], possibly due to CD81 localization in tetraspanin-enriched microdomains [46]. Interestingly, CD81 is a putative receptor for hepatitis C virus [47]. CD81 role in parasite invasion has been suggested to occur through

association with other host molecular partners. The host protein SR-BI by mediating cholesterol uptake facilitates CD81 organization into tetraspanin-enriched microdomains, thereby enhancing parasite invasion [48, 49]. A host kinase, PKC ζ (PKCzeta), has been shown to influence infection *in vitro* as well as *in vivo*, since its downregulation impairs hepatocyte invasion [50].

1.3.2.2.3 Parasite development in hepatocytes

With the final hepatocyte invasion, each *Plasmodium* sporozoite, by invagination of the plasma membrane is internalized within the parasitophorous vacuole. Here, the parasite undergoes extensive nuclear division to form a multinucleated syncytium, the schizont (FIGURE 4C). Each nucleus is later surrounded by a membrane. Exoerythrocytic schizogony represents one of the most remarkable replication rates in the eukaryotic world culminating in the production of 10 000 to 30 000 merozoites, the red blood cell infectious forms that are released in the bloodstream. The astonishing replication rate in the liver can only be achieved through an intimate and extensive connection between parasite and hepatocytes. Liver cells are one of the most metabolically active cells, representing an ideal source of metabolites to support parasite growth. However, still relatively little is known about the complex cellular and molecular interactions between parasite and host during this phase of *Plasmodium* life cycle.

Infective sporozoites were shown to induce the expression of two parasite genes termed *UIS3* and *UIS4* (*UIS* stands for *upregulated in infective sporozoites*) [51]. In the liver, *UIS3* and *UIS4*, two transmembrane proteins, are localized to the parasitophorous vacuole membrane (PVM) within the first hours of parasite development. Its depletion inhibits the establishment of a successful *in vivo* liver infection, since although able to invade no mutant parasite is detected within 48 hours after sporozoite injection ([52],[53]). *UIS4* role during the parasite liver stage development is still unknown. On the other hand, *UIS3* has been suggested to bind to the liver fatty acid binding protein (L-FABP) [54, 55], an interaction that possibly provides the parasite with a pathway to obtain host cell lipids. However, this interaction has been challenged by a recent study using nuclear magnetic resonance (NMR) spectroscopy [56]. Further work is necessary to elucidate this controversial data. Together with *UIS3* and *UIS4*, the parasite exported protein 1 (*EXP1*) is also a PVM protein detected in late liver stage developed parasites ([57],[58],[59]). Another parasite protein that has a role during liver development, but not only, is the previously mentioned CSP. CSP is translocated to the hepatocyte cytoplasm and imported to the host cell nucleus within the first 4 hours after parasite invasion. There, CSP has been shown to inhibit the

translocation of NF- κ B to the nucleus [60].

The technical difficulties concerning *Plasmodium* liver stage research has been the main cause for our lack of knowledge of this stage. However, understanding the hepatocyte role during infection will provide us the insight into the processes that are vital for *Plasmodium* development and merozoite formation. A major breakthrough on this matter has been recently achieved by Albuquerque *et al.* who, based on a genome-wide microarray analysis of host cells infected with *P. berghei* sporozoites, identified a coordinated and sequential set of biological pathways in the host cell that are modulated with infection [61]. Importantly, several metabolic pathways, such as lipid metabolism and glucose metabolism are significantly altered during hepatic infection. Together with these pathways, aminoacid metabolism and oxidative stress pathways are also modulated with infection (FIGURE 5), as well as activation of inflammatory responses, which were later confirmed in an *in vivo* model of hepatic infection by microarray analysis [62]. During the different stages of *Plasmodium* life cycle immune responses are triggered in order to control the infection. Our current knowledge of this aspect of *Plasmodium* infection is summarized in FIGURE 6.

Thus, *Plasmodium* liver infection modulates the transcriptional landscape of hepatocytes, leading to changes in its metabolic signature. The impact of these profound changes in hepatocytes, rather if they are directly induced by the parasite or a consequence of the host cell response to infection and, finally, how it impacts infection are crucial questions that remain largely unanswered.

1.3.2.2.4 Exit the liver to the blood

Ceased the replication process inside the hepatocyte, the parasitophorous vacuole contains the membrane-bound infective merozoites ready to be released into the bloodstream and invade red blood cells. In late schizont stages, merozoite surface protein 1 (MSP1) is expressed and incorporated in the schizont plasma membrane that, through continues invaginations, forms the merozoites membrane [63]. During the release process, the PVM disrupts and the parasites are released into the host cytoplasm. The merozoites are then released into the bloodstream inside merozoite-filled vesicles, called merosomes (FIGURE 4D). These small vesicles, whose membrane is originated from the host cell membrane, are release into the liver sinusoids. It has been proposed that the membrane host origin assures that merosomes are not recognized by Kupffer cells, and so can safely transport the merozoites into the bloodstream [57]. At last, after passing the liver sinusoids and the heart intact, merosomes are sequestered in the lung capillaries. Here, the merosome membrane disintegrates and the free infective-merozoites go through the lung circulation and begin the blood stage of *Plasmodium* infection [64].

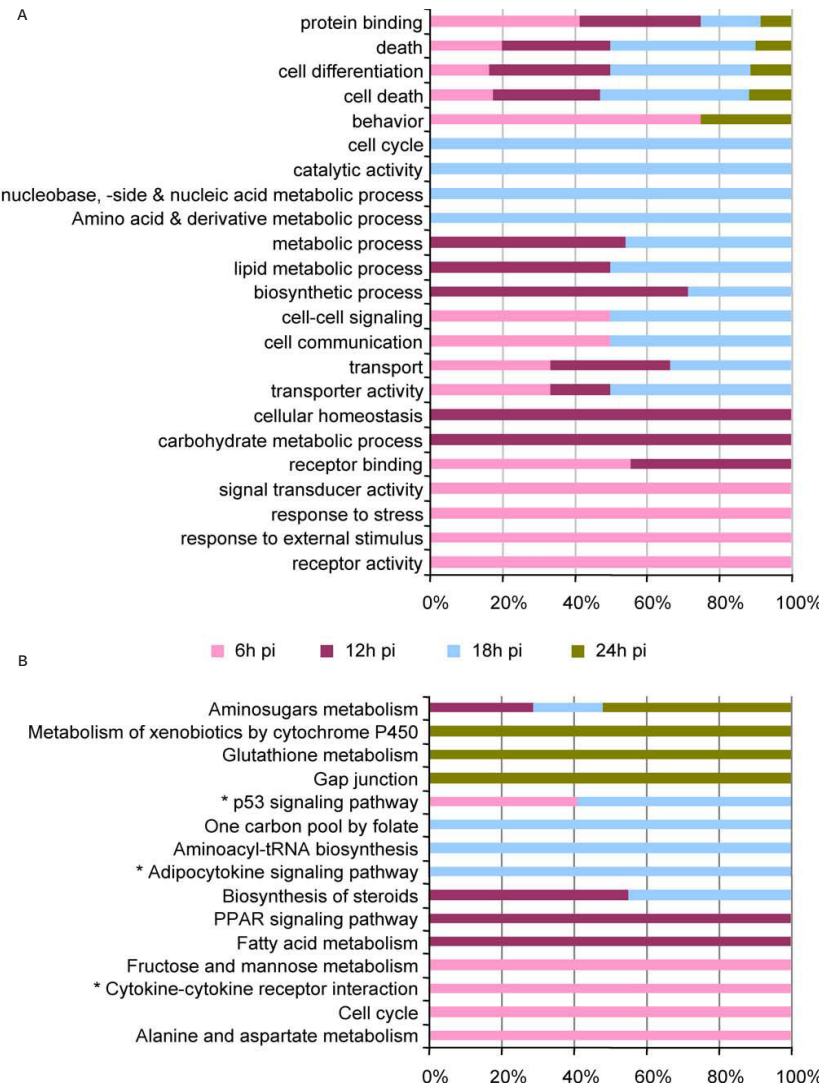


FIGURE 5
Microarray analysis identified several metabolic pathways as significantly altered during infection.
Gene Ontology enrichment analysis ($p < 0.01$) based on GOSlim. Genes found significant in the analysis were grouped by function following GO annotation. The stacked bars show the contribution of each time point across categories (A) KEGG metabolic pathway enrichment analysis calculated using the differentially expressed genes having a KEGG annotation (B) (Adapted from Albuquerque, et al 2009).

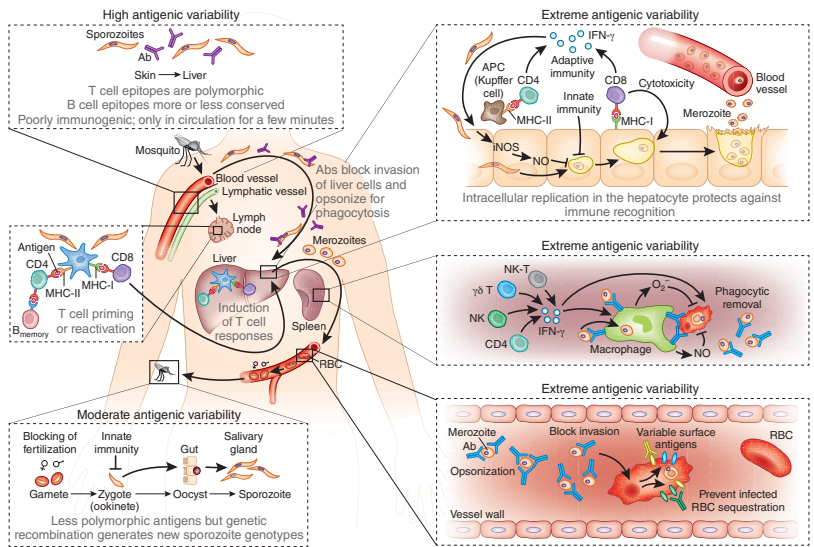


FIGURE 6
***Plasmodium* life cycle and the main immune responses that control the parasite at each stage.**

With the bite of an infected mosquito, sporozoites injected into the skin are drained to the lymph nodes (here they activate T and B cells) or to the liver where they invade hepatocytes. Antibodies against sporozoites neutralize sporozoites and/or block invasion of hepatocytes. This response is, however, inefficient since sporozoites are in circulation only for a few minutes (sporozoites take typically 10–15 minutes to reach the liver and merozoites invade a red blood cell in less than 30 seconds). In the blood stage, merozoite-specific antibodies agglutinate and opsonise the parasite and can block invasion of red blood cells. Antibodies drive the opsonisation and agglutination of infected red blood cells (iRBCs) preventing their sequestration in blood vessels of small calibre. IFN-γ producing lymphocytes activate macrophages enhancing opsonised merozoites and iRBCs phagocytosis. Antibody and complement to gametocytes are taken up in the blood meal and mediate the lysis of gametocytes, thus preventing its fertilization and further development in the mosquito. **Liver immune responses** – immune responses against *Plasmodium* liver stage depend mainly on the effector CD8⁺T cells, with IFN-γ being the critical

effector molecule, and interleukin-4 (IL-4) secreting CD4⁺T cells required for induction of the CD8⁺T cell responses. Additionally, components of the innate immune system, including gamma-delta T cells (γδ T), natural killer cells (NK) and natural killer T cells (NKT) have also been implicated. While *Plasmodium* erythrocytic stages induce potent innate immune responses, the role of innate immunity to liver stage parasites remains largely unknown. Whether parasites are detected in the liver by innate immune mechanisms and how it impacts the outcome of liver infection are important questions to be addressed (Adapted from Riley and Stewart, 2013).

1.4 Models for *Plasmodium* liver stage infection

Plasmodium liver stage of infection still remains a “black box” in comparison to other life cycle stages. Identified in 1948, technical constraints have been a major obstacle in the study of *Plasmodium* liver infection [65]. Although the establishment of human primary hepatocytes in culture has increased, which allow *Plasmodium falciparum* infection, the culture conditions are still very demanding and difficult to implement. Human hepatocyte cell lines, HepG2 [66] and HC04 [67], have been shown to support *Plasmodium vivax* and the latest being the only cell line that supports *Plasmodium falciparum* *in vitro* liver infection [67]. However, a particular weakness of these procedures is the need for mosquitoes infected with human parasites, which demands specific safety protocols and infrastructures that are expensive and scarcely used. As such, one of the important breakthroughs in the research of *Plasmodium* infection was the establishment of mouse models. For the liver stage field particularly, rodent *Plasmodium* species as *Plasmodium berghei* and *Plasmodium yoelli* have been used extensively. These parasite strains are also able to infect mouse primary hepatocytes, as well as mouse hepatoma cell lines as Hepa 1-6 [68]. *Plasmodium berghei*, although a rodent parasite, successfully infects human hepatoma cell lines, as Huh7 [69] and HepG2 [70]. Here, however, the maintenance of mosquito colonies is still mandatory.

An intrinsic characteristic of hepatic infection by *Plasmodium* is its low infectivity: only a few sporozoites are delivered in a single infected mosquito bite [71]. In *in vitro* and *ex vivo* infections the percentage of parasitized cells is extremely low (< 5%) [69] and even increasing the number of sporozoites added to the culture does not lead to a linear enhancement of infection. *In vivo* systems, as mouse models, while the number of sporozoites can be greatly increased if we pass from mosquito bites, the physiological way of parasite transmission, to intravenously sporozoite injection (the most efficient way of delivering sporozoites and have higher liver parasite loads) only a small proportion of the total number of hepatocytes is infected. Parasites that express GFP throughout its life cycle, including the liver stages, have been established in order to help researchers circumvent this particular issue by allowing, for example, the use of fluorescence-activated cell-sorting of infected cells and thus enrich the pool of parasitized cells [69].

Hence, the study of *Plasmodium* liver stage has been *shadowed* by its own intrinsic biology and only recently, owing to technological advances, we start to shed light on its events.

2 THE LIVER

Plasmodium targets the liver as the final address to engage an astonishing replication process. The liver is a vital organ which fulfils crucial metabolic and clearance functions due to its main cells – the hepatocytes. Hepatocytes are one of the most impressive multi-taskers cells in the body, being responsible for protein synthesis, metabolism of lipids, peptides, carbohydrates and nutrients, such as iron [72]. To perform its diverse metabolic functions, hepatocytes are highly enriched in an organelle - the endoplasmic reticulum [73]. Interestingly, recent research has suggested that in high metabolically active cells, such as hepatocytes, the endoplasmic reticulum is essential in the maintenance of cellular homeostasis and several infectious and metabolic diseases are characterized by the activation of signalling pathways steaming from the ER. In the following sections I will present the endoplasmic reticulum main functions and how perturbations to it are connected to disease, mainly in the liver.

2.1 The Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an extensive and continuous membrane network that functions as a central regulator of multiple cellular processes. The ER is the first organelle of the secretory pathway. In its lumen, nascent secretory and transmembrane proteins, together approximately 30% of the cellular proteome, fold and mature before being delivered to other organelles, cell surface or released extracellularly ([74],[75]). Also, the highest intracellular concentrations of calcium are found in the ER, making it the major signal-transducing organelle in the cell [76]. At the cytosolic face of the ER membrane occur lipogenic reactions for the synthesis of triacylglycerols, sterols, ceramides, and the majority of cellular phospholipids, as also as fatty acids desaturation [77]. Enzymes involved in gluconeogenesis and specialized pathways of fatty acid oxidation are also housed at the ER. The ER contributes to the biogenesis of peroxisomes [78], lipid droplets [79] and autophagic membranes [80] and is continuous with the nuclear envelope. It closely contacts almost every membrane-bound organelle in the cell, including mitochondria, endosomes, the Golgi apparatus and peroxisomes, as well as the plasma membrane [81].

This physical and functional interconnectivity facilitates the bidirectional transfer of lipids, calcium and other molecules, and conveys to the ER a central role as regulator of numerous aspects of cellular and organism homeostasis.

The ER must sense and integrate diverse and constantly changing physi-

ological inputs in order to maintain cellular homeostasis. Such regulatory processes can only be achieved through a crosstalk between cellular signalling pathways and the ER. The first sign to the existence of such intracellular signalling events was found in 1988. Kozutsumi *et al* observed that pharmacological and genetic manipulations that increase the burden of unfolded proteins in the ER cause a transcriptional induction of ER-resident chaperone-encoding genes ([82],[83]). This intracellular signalling pathway became known as the **Unfolded Protein Response** (UPR). Described originally as a stress response, the principles of UPR activation are relatively well defined. Perturbations that require an increase in the protein folding machinery but that exceed ER's capacity lead to the accumulation of unfolded and misfolded proteins in the ER lumen. The resulting loss of ER-homeostasis (called ER stress) activates the UPR that consists of fast but also long term adaptations. The immediate response, a transient adaptation, is the reduction of protein entering the ER; changes in gene expression, on the other hand, promote long-term adaptation and, if homeostasis cannot be re-established, cell death is eventually triggered (for a detailed review see [84]). The ER is responsible for a broad spectrum of processes; therefore calcium depletion, oxidative stress, hypoxia, glucose deprivation, viral infections as well as metabolic stimulation can cause ER stress and activate the UPR [85]. Our current understanding about the diversity of molecular signalling mechanisms that coordinate the complex UPR is the subject for the next sections, focusing on mammalian UPR.

2.2 The mammalian UPR

Three main branches of the mammalian UPR have been identified. The branches are activated in parallel and use unique mechanisms of signal transduction. Each branch is defined by a class of ER transmembrane proteins: IRE1 (inositol requiring protein 1), PERK (protein kinase RNA (PKR)-like ER kinase) and ATF6 (activating transcription factor 6). Each of these factors has an ER luminal portion that senses the protein-folding environment in the ER and transmits this information across the ER membrane to the cytosol. The IRE1 branch is the evolutionary most conserved branch of the UPR and the only identified ER stress sensor in yeast [86]. Later in evolution the PERK and ATF6 branches were added to metazoan cells. The complexity of mammalian UPR has evolved to a tissue and cell type specialization – IRE1 and ATF6 have paralogs; ATF6 shares homology with two additional ER transmembrane proteins – cAMP responsive element-binding protein, hepatocyte specific (CREBH) in liver hepatocytes and old astrocyte specifically induced substance (OASIS) in brain astrocytes [87].

2.2.1 The IRE1 α -XBP1 pathway

IRE1, the most conserved and only branch of the UPR in lower eukaryotes, is the first stress transducer identified and the best-studied UPR branch due to its presence in yeast (FIGURE 7). In mammals there are two IRE1 homologues: Ire1 α is expressed ubiquitously, whereas IRE1 β expression is confined to the intestinal epithelium.

Ire1 α is a type 1 ER-resident transmembrane protein (with an ER-luminal domain that senses unfolded proteins in the ER and a cytoplasmic portion that has both a serine/threonine kinase and an endoribonuclease (RNase) domain ([88],[89]). Via the coordinated action of its cytosolic effector domains, Ire1 α signals UPR in a transcriptional program. Two models have been proposed for the activation of Ire1 α – one posits a competition-binding model whereas an alternative model proposes that un- and misfolded proteins bind to the IRE1 luminal domain directly (see BOX 1 for a more detailed discussion). As unfolded proteins accumulate, the luminal domain of Ire1 α rearranges allowing lateral oligomerization in the ER membrane, thereby concentrating the covalently tethered kinase/RNase domains on the cytosolic side of the membrane [90]. This concentration event is thought to provide the primary switch leading to Ire1 α activation. Oligomerization opens the kinase domain allowing ATP to enter and phosphorylates the activation loop in trans (trans-autophosphorylation), which in turn activates its RNase activity ([90, 91]).

When activated, IRE1 α induces the unconventional splicing of its only known substrate – an mRNA encoding a transcription factor called, in metazoans, XBP1 (X-box binding protein 1) ([92],[93]).

Splicing of the precursor, unspliced *XBP1* mRNA (XBP1u) encodes an active transcription factor – the spliced XBP1 (XBP1s). In mammalian cells, the excision of a 26 base intron from unspliced *XBP1* mRNA results in a translational frameshift that induces the conversion of the 267 amino acids unspliced form to a spliced form of 371 amino acids (comprising the original N-terminal DNA-binding domain plus an additional, potent transactivation domain in the C terminus). While the DNA-binding domain (DBD) and the transcriptional activation domain (AD) are encoded in separate open reading frames, the protein translated from *XBP1u* mRNA has only the DBD and cannot activate transcription. However, the splicing event in response to UPR removes the intron and joins the two ORFs (open reading frames) so that XBP1s has both DBD and AD [92]. XBP1s binds to the UPR element (UPRE) and to ER stress-response elements I and II (ERSE-I and ERSE-II) in the promoter regions of a broad spectrum of UPR-related genes ([94],[95],[96]). XBP1s binding induces the upregulation of factors involved

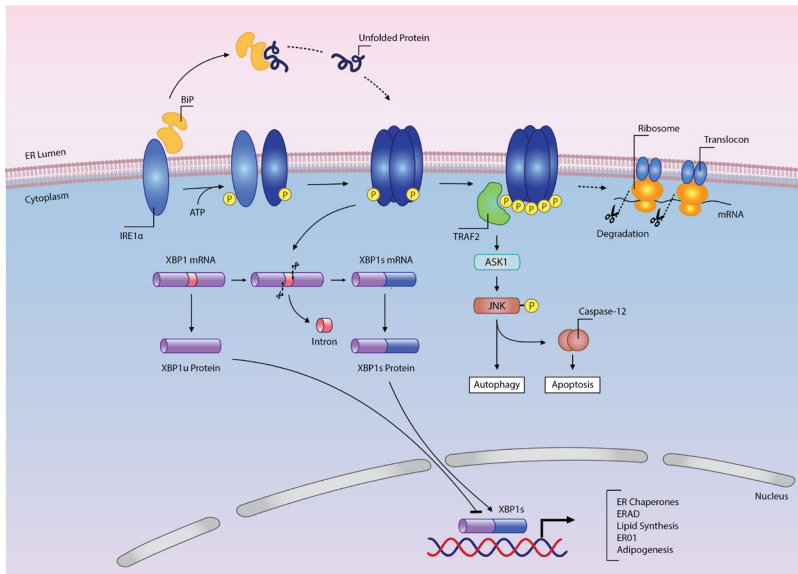
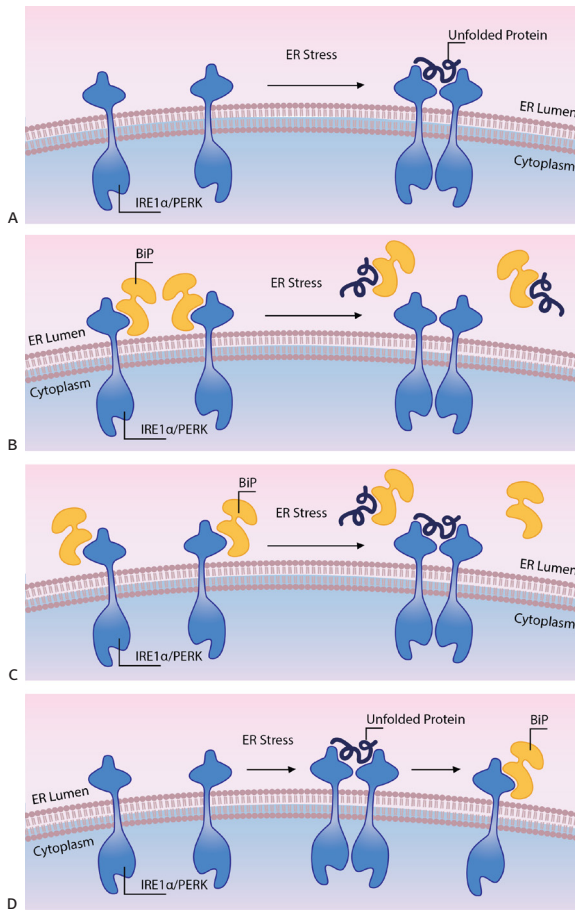


FIGURE 7
Signalling by IRE1.

In stressed cells, Inositol-requiring protein-1 (IRE1) oligomerizes in the plane of the endoplasmic reticulum (ER) allowing for trans-autophosphorylation in its cytosolic kinase domain and activating its RNase activity. IRE1 mediates a sequence-specific cleavage of a single known mRNA (X-box binding protein-1 (XBP1) in higher eukaryotes, excising a small RNA fragment (intron). The two ends of the mRNA are ligated (the ligase identity in metazoans is unknown) leading to a frame shift in the coding sequence (shown in the figure as a colour change from purple to blue after removal of the intron). Spliced XBP1 mRNA encodes a potent transcriptional activator (XBP1s), whereas the unspliced XBP1 mRNA encodes XBP1u, an inhibitor of the unfolded protein response (UPR). XBP1s regulates a subset of UPR genes that promote ER-associated degradation (ERAD) of misfolded proteins and ER biogenesis. In addition to the transcriptional remodeling of the ER folding environment, IRE1 is also involved in the activation of cell death pathways in response to prolonged ER stress. Phosphorylated IRE1 associates with tumor necrosis associated factor 2 (TRAF2) and activates the c-Jun N-terminal kinase (JNK) pathway via the apoptosis signaling-regulating kinase (ASK1), which controls

apoptosis through caspase 12 activation. Additionally, IRE1 is linked to a nonspecific RNase activity that degrades mRNA localized to the ER membrane termed regulated IRE1-dependent decay of mRNAs (RIDD), leading to a global reduction in proteins imported into the ER lumen (see review by Ron and Walter, 2007).



BOX 1

How is ER stress sensed?

The endoplasmic reticulum (ER) stress transducers inositol-requiring protein-1 (IRE1) and protein kinase RNA (PKR)-like ER kinase (PERK) are enzymes whose activation is linked to perturbed protein folding in the ER. The ER lumen domain of both IRE1 and PERK are experimentally interchangeable and evolutionarily related. Different models for how these domains sense the unfolded protein load have been proposed (see review by Ron and Walter, 2007; Hetz *et al*, 2011). A direct recognition model was proposed where, based on structural studies of the ER luminal domain of both human and yeast IRE1, misfolded proteins may actually bind the NH₂-terminal region of IRE1 promoting its oligomerization (A). The indirect recognition model suggests that the ER chaperone BiP binds to the luminal domain

of IRE1α maintaining it in an inactive state as a monomer. In cells undergoing ER stress, BiP is released and binds to unfolded proteins allowing for IRE1α activation through its oligomerization and autophosphorylation (B). However, mutagenesis of BiP binding site did not change the ability for IRE1 to detect unfolded proteins, in yeast. A third, hybrid recognition model proposes that both BiP dissociation and peptide binding cause sensor activation (C). Recently, a new model has been proposed where three pools of IRE1p may exist: an inactive subpopulation in equilibrium with an active unfolded protein-bound pool and a third inactive set sequestered by BiP for inactivation. In this model, BiP binding to or release from IRE1p does not activate the UPR, but it may adjust the sensitivity and dynamics of IRE1p activity (D).

in protein folding, protein entry into the ER, ER-associated degradation (ERAD) pathway and protein quality control ([97],[98]). In addition, XBP1s regulates the secretory pathway expansion by activating phospholipid biosynthesis and ER/Golgi biogenesis ([98],[99]).

Opposite to what is observed in yeast, the unspliced *XBP1* mRNA in metazoans is translated into protein. But what is the function of XBP1u? Recent studies have shown that XBP1u expression is increased during the recovery phase of the UPR and binds and sequesters XBP1s from the nucleus, enhancing its degradation by the proteasome. These actions lead to the shutdown of the UPR-transcriptional programme ([100],[101]). These findings indicate that XBP1u is a negative feedback regulator of mammalian UPR. Thus, XBP1 mRNA autoregulates its activity by producing a repressor and activator in the forms of unspliced and spliced mRNA, respectively.

IRE1-mediated splicing of *XBP1* mRNA is remarkably specific and distinct from a parallel mode of RNA degradation dependent on IRE1. This pathway – termed regulated IRE1-dependent decay of mRNAs (RIDD) – is responsible for the degradation of ER-localized mRNAs in *Drosophila* [102] and mammalian cells [103] in a stress-dependent manner, likely to reduce protein influx as well the load of unfolded proteins into the ER lumen.

In mammals, Ire1 α has additional functions in cell signalling besides its nucleolytic activity. The cytosolic domain of Ire1 α activates the apoptosis signal-regulating kinase 1 (ASK1) and the stress-induced Jun N-terminal kinase (JNK) ([104],[105]); it also activates p38 and ERK [106] kinases, as well as NF- κ B pathways [107]. The function of these UPR-signalling branches is, however, poorly understood. Recently, Ire1 α was proposed to control autophagy under ER stress conditions through activation of JNK [108]. In cells undergoing ER stress autophagy may serve as a mechanism to eliminate damaged organelles and proteins aggregates ([109],[110],[111]). A genomic screen in fly cells demonstrated that the knockdown of UPR components, including XBP1, increases basal autophagy levels [112] and autophagy defective cells have upregulation of essential ER chaperones [113], suggesting that autophagy and UPR pathways are connected to a homeostatic balance.

These data show that Ire1 α has a dual role in ER stress responses: (1) regulating adaptation to stress through XBP1 and (2) activating apoptosis in cells irreversibly damaged by the activation of the JNK/ASK1 pathway.

2.2.2 The PERK-eIF2 α pathway

The second branch of the UPR is mediated by PERK, a type I ER-resident transmembrane protein with a cytosolic serine/threonine kinase domain similar to IRE1 (FIGURE 8). Although exhibiting limited sequence homology, both PERK and IRE1 share ER luminal stress-sensing domains that

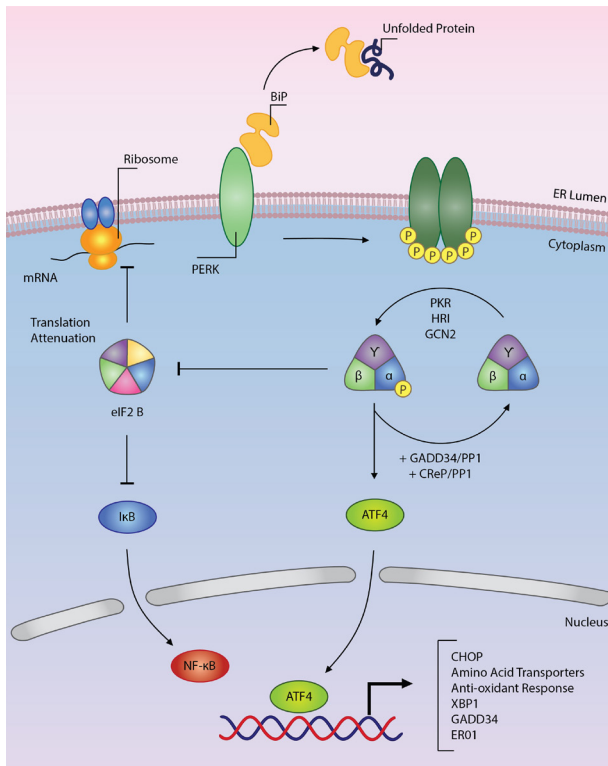


FIGURE 8
Signalling by PERK.

In response to endoplasmic reticulum (ER) stress, protein kinase RNA (PKR)-like ER kinase (PERK), similar to inositol-requiring protein-1 (IRE1), oligomerizes in the plane of the membrane and is activated by trans-autophosphorylation. Extensive further phosphorylation facilitates the recruitment of its substrate. PERK, once activated, phosphorylates the α subunit of eukaryotic translation initiation factor-2 (eIF2) on Ser51, its only known substrate, inhibiting the eIF2B complex (pentameric guanine nucleotide exchange factor) of recycling eIF2 to its active GTP-bound form. Hence, PERK activation results in reduced activities of eIF2B and the eIF2. At high levels of eIF2 α phosphorylation, NF- κ B signaling is also activated (through relief of I κ B inhibition), potentially promoting cell survival and an inflammatory response. Besides PERK, other eIF2 kinases (PKR, haem-regulated inhibitor kinase (HRI) and general control non-derepressible-2 (GCN2)) can phosphorylate eIF2 α and activate this pathway

independently of ER stress, and so this branch of the UPR is termed the integrated stress response (ISR). eIF2 α phosphorylation results in lower global protein translation, reducing the ER unfolded protein load but it also affects gene transcription by selectively inducing the translation of activating transcription factor-4 (ATF4). The ISR activates genes that encode amino-acid transporters and genes that protect against oxidative stress, and it contributes to the transcriptional activation of XBP1. Also activated by ATF4 is the transcription factor CHOP (C/EBP-homologous protein) whose target genes include GADD34 (growth arrest and DNA damage-inducible protein-34), a regulatory subunit of phosphatase PP1, that dephosphorylates eIF2 α and terminates signaling in the ISR, and ER oxidase-1 (ERO1), which is required for disulphide bond formation in protein folding. A constitutive phosphatase CReP (constitutive repressor of eIF2 α phosphorylation) is responsible for the control of eIF2 phosphorylation in non stress conditions (see review by Ron and Walter, 2007).

are phylogenetically related, are similar in structure and function, and are experimentally interchangeable; the luminal domain of human PERK can replace the protein misfolding sensor of the luminal domain of yeast IRE1p, despite the absence of a PERK gene in yeast [114]. When activated upon ER stress, PERK undergoes oligomerization and *trans*-autophosphorylation. PERK then phosphorylates the α -subunit of eukaryotic translation factor-2 (eIF2 α) at Ser51. This inhibits the activation of eIF2 by inhibiting the complex that recycles eIF2 to its active GTP-bound form. Decreasing eIF2 activation results in lower levels of translation initiation (FIGURE 9) and reduces the ER protein-folding load [115]. On the other hand, eIF2 α phosphorylation activates translation of mRNAs encoding several short upstream open reading frames (uORFs) [116]. Phosphorylation of eIF2 α inhibits eIF2 activation and decreases the concentration of the 43S preinitiation complex. At low levels of eIF2 α phosphorylation, uORFs are efficiently translated and consequently no translation occurs of the downstream ORF. When phosphorylation of eIF2 α is high, several uORFs are scanned before the small ribosomal is loaded again with the ternary eIF2-GTP-Met-tRNA_i^{Met} complex, which allows for translation of the downstream ORF. This mechanism of translational control in response to ER stress selectively induces translation of *Atf4* ([117],[118]) and *Cat-1* mRNAs [119]. ATF4 (activating transcription factor 4) is a 39 kDa bZIP transcription factor that induces transcription of genes encoding ER chaperones and UPR-associated transcription factors, including XBP1. Furthermore, ATF4 induction of amino acid biosynthesis and transport, fostering anti-oxidative stress responses and even stimulating the expression of autophagy genes alleviates ER stress and improves cellular homeostasis ([118],[120]).

One of the important transcripts activated by ATF4 is CHOP (C/EBP homologous protein), a 29 kDa bZIP transcription factor, and an important mediator in ER stress induced apoptosis. Under severe ER stress, CHOP activates apoptosis by regulating the expression of a multitude of pro-apoptotic factors, such as Bim (a pro-apoptotic member of the BCL-2 family) [121] and TRB3 (Tribbles homolog 3) [122], while it inhibits the anti-apoptotic protein BCL-2 [123]. Thus, the PERK branch of the UPR has both a strong protective role, when submitted to modest levels of ER stress, but can also trigger cell death. The PERK branch duality in outcomes shows that the level of eIF2 α phosphorylation must be tightly regulated. Indeed, phosphorylated eIF2 α is subject to negative regulation: CHOP induces transcription of *Gadd34*, which encodes a regulatory subunit of the protein phosphatase PP1c that counteracts PERK by dephosphorylating eIF2 α ([124],[125]). While GADD34 is induced as part of the gene expression programme activated by eIF2 α phosphorylation, CReP (constitutive repressor of eIF2 α phosphorylation) is constitutively expressed

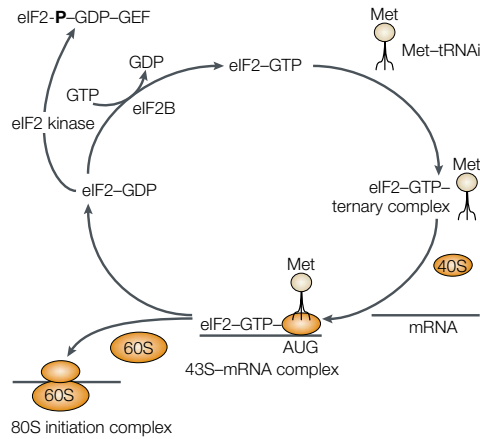


FIGURE 9
Regulation of translation initiation through eIF2 phosphorylation.

Eukaryotic translation initiation factor 2 (eIF2) is a heterotrimeric protein that is required to bring the initiator methionyl-transfer RNA (Met-tRNAⁱ) to the 40S ribosome. Polypeptide-chain synthesis starts when the ternary complex of eIF2, GTP and initiator Met-tRNA binds to the 40S ribosomal subunit to generate a 43S species. After mRNA binds to the former complex, the 60S ribosomal subunit joins to form the 80S initiation complex with the concomitant hydrolysis of GTP to GDP. For eIF2 to promote another round of initiation, GDP bound to eIF2 must be exchanged for GTP in a reaction that is catalysed by the guanine nucleotide exchange factor eIF2B. Phosphorylation of the α -subunit of eIF2 (eIF2 α) on serine 51 stabilizes the eIF2-GDP-eIF2B complex and prevents the GDP-GTP exchange reaction. Because eIF2B is required for the exchange of GDP for GTP, and since the cellular levels of eIF2B are roughly one-tenth of the levels

of eIF2, the exchange process is inhibited when only a fraction (20–30%) of eIF2 α is phosphorylated and in an inactive complex with eIF2B. Phosphorylation of eIF2 α , therefore, immediately reduces the level of functional eIF2, and limits initiation events on all cellular mRNAs within the cell. So, control through reversible phosphorylation of eIF2 α provides the cell with an efficient and rapid means to respond to various different stress stimuli. (Adapted from Kaufman et al 2002).

and controls baseline eIF2 α dephosphorylation [126].

In mammals, other three additional kinases, each responding to a different stress, induce eIF2 α phosphorylation and activate a common set of target genes (FIGURE 8). Due to this integrative feature, the signalling downstream of eIF2 α was termed the integrated stress response (ISR) [127].

2.2.3 The ATF6 pathway

A third branch of the mammalian UPR is mediated by ATF6, a type II ER transmembrane protein encoding a bZIP transcription factor in its cytosolic domain and bearing a large ER-lumenal domain [128]. ATF6 is initially synthesized as an inactive precursor tethered to the ER membrane. Upon accumulation of unfolded proteins, ATF6 is packaged into transport vesicles that detach from the ER and are delivered to the Golgi apparatus [129]. There, it is cleaved sequentially by 2 Golgi-resident proteases: S1P (site 1 protease) removes the lumenal domain, while S2P (site 2 protease) removes the intramembrane region ([128],[130]). The N-terminal cytosolic DNA-binding fragment released (ATF6-N) migrates to the nucleus to activate UPR target genes (FIGURE 10). The process by which transmembrane proteins are cleaved to liberate cytosolic fragments is termed regulated intramembrane proteolysis (RIP) and this mechanism of regulation is shared by SREBPs (sterol regulatory element-binding-proteins) in mammalian cells.

Mammals have two ATF6 homologues, ATF6 α and ATF6 β , which are expressed ubiquitously. Once activated, ATF6 α binds to ERSE motifs in promoters to induce transcription of genes that encode for ER chaperone genes, such as BiP (a chaperone of the heat shock protein HSP70 family), PDI (protein disulfide isomerase) and GRP94 (glucose-regulated protein 94), but also XBP1 [92]. ATF6 α can heterodimerize with XBP1 in response to ER stress and induce the expression of ERAD components, as EDEM (ER degradation enhancer, mannosidase alpha-like 1), HERPUD1 (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1) and HRD1 (ubiquitin-protein ligase/synoviolin) ([131],[132],[133]). To date, ATF6 β regulated genes remain to be found.

2.2.4 CREBH – a liver specific ER stress transcription factor

Recently, a number of ATF6 α -related proteins were identified suggesting that the three-arm branches canonical UPR might not be complete. To date, five additional proteins – Luman (CREB3), Oasis (CREB3L1), BBF2H7 (CREB3L2), CREBH (CREB3L3) and Tisp40 (CREB4, CREB3L4) – have been identified as RIP-regulated bZIP transcription factors, as ATF6 α , and have all been implicated in the ER stress response [134].

CREBH is a liver-specific transcription factor that is proteolytically activated

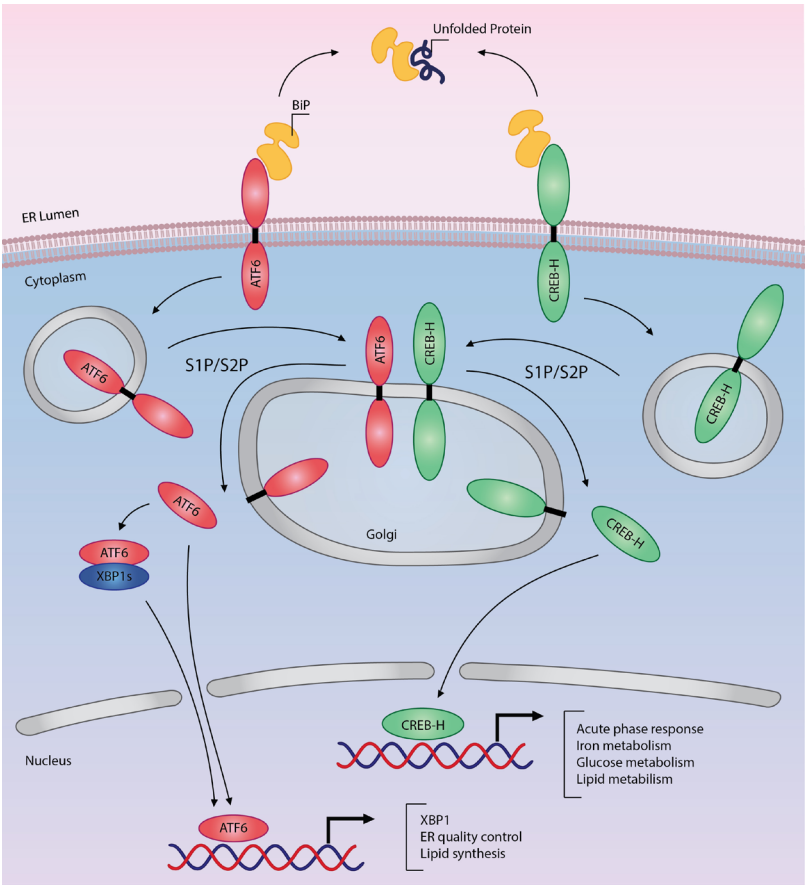


FIGURE 10
Signalling by ATF6 and CREBH.
 Activating transcription factor-6 (ATF6) and cyclic AMP response element binding protein hepatocyte (CREBH) are transmembrane proteins with a cytoplasmic portion that can bind to DNA and activate target genes. Upon accumulation of unfolded proteins, ATF6 and CREBH are delivered to the Golgi apparatus and are subject to consecutive cleavage, first by the luminal site 1 protease (S1P) and then the intra-membrane site 2 protease (S2P), which liberates the cytosolic effector portions of the proteins from the membrane and allows their import into the nucleus. ATF6 may heterodimerize with XBP1 before trafficking to the nucleus to activate genes encoding ER chaperones. While ATF6 activates UPR target genes, as ER chaperones and XBP1 and is involved in lipid homeostasis, CREBH activates acute-phase response genes

that encode secreted proteins involved in inflammation and is a regulator of iron metabolism in the liver. Also, CREBH has been recently implicated in lipid and glucose metabolism (see review by Ron and Walter, 2007).

upon ER stress (FIGURE 10). Unlike ATF6 α , CREBH is not involved in transcriptional induction of UPR genes. Instead, CREBH induces the expression of genes from the innate immune response – the acute phase response [135]. Equally important to note, CREBH is the mediator of ER stress-regulated iron homeostasis [136] and recent studies demonstrated that CREBH-mediated transcription is important in regulation of gluconeogenesis [137] and lipid metabolism in the liver [138].

The existence of a family of ATF6 α -like proteins that are differentially represented among tissues and are activated by different profiles suggests that, in mammals, the UPR has evolved additional factors that allow fine-tuned responses that are related to specific cells and tissues, but also the encountered stress factor as well as the duration of the stress.

2.3 ER, Metabolism and UPR

Hitherto, I briefly reviewed the cellular functions of the ER and how, in response to ER stress, cells activate the UPR to re-establish cellular homeostasis. However, the physiological consequences due to ER dysfunction cannot be solely attributed to cell-autonomous responses to ensure proper protein folding. Due to its regulatory panoply, the ER can be viewed as a metabolic compartment capable of establishing specific functional links with metabolic responses. As so, UPR is instrumental in maintaining the homeostasis of organisms by regulating full organs, including the liver. In the following section our current understanding of how UPR integrates lipid and glucose metabolism as well as iron homeostasis is presented. For the purpose of this work the focus will primarily be on liver metabolism.

2.3.1 UPR in Lipid Metabolism

The ER is the main lipid biosynthetic organelle and is responsible for cholesterol and fatty acids, as well as glycerolipids and triglycerides synthesis. The primary mechanisms of lipid metabolism in the ER are depicted in FIGURE 11. Although the ER is the main site of cholesterol synthesis, this lipid is rapidly transported to other organelles. Owing to its role to membrane rigidity, cholesterol is tightly regulated by pathways that sense the level of cholesterol and its derivatives within the ER membrane and induce signals that control cholesterol synthesis and clearance.

SREBP-2 is the main transcription factor responsible for regulating the cholesterol biosynthetic pathways. SREBP-2 is resident in the ER and belongs to the SREBP (sterol regulatory element-binding-proteins) family of transcription factors. The three mammalian isoforms, SREBP1a, -1c, and -2 are anchored in the ER membrane bound to SCAP (SREBP cleavage-activating protein). In response to low sterol levels or insulin, the SCAP-SREBP2 com-

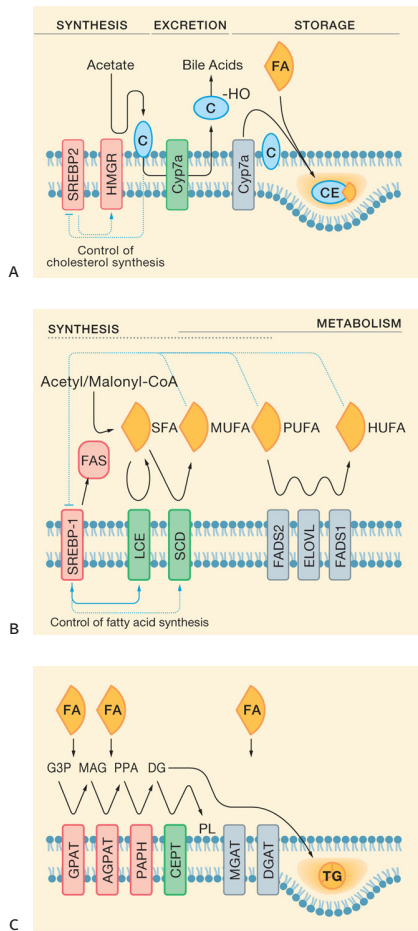


FIGURE 11

Lipid metabolism at the Endoplasmic Reticulum

A – Key metabolic processes in cholesterol metabolism occurring at the ER include control of cholesterol synthesis via SREBP release and upregulation of cholesterol synthesis enzymes including HMG-CoA reductase (HMGCR), oxidation of excess membrane cholesterol by Cyp7a, a key step in liberating cholesterol for bile acid production and secretion, and the acylation of cholesterol into cholesterol esters by acyl-CoA:acyltransferase (ACAT), which, like DGAT, creates a highly hydrophobic entity that seeds the formation of lipid droplets within the membrane phase.

B – Key activities in fatty acid metabolism include synthesis of saturated fatty acids (SFA) via fatty acid synthase (FAS) and their elongation and desaturation to longer-chain SFA and monounsaturated fatty acids (MUFA) by the long-chain elongase (LCE) and stearoyl-CoA desaturase (SCD), respectively. Synthesis of fatty acids is controlled by SREBP-1. Essential (polyunsaturated, PUFA) fatty acids are metabolized by desaturation and elongation of highly unsaturated fatty acids (HUFA) by desaturases (FADS) and elongases (ELOVL), respectively.

C – Key activities in the synthesis of glycerolipids including phospholipids (PL) and triglycerides (TG) include glycerol-3-phosphate acyltransferase (GPAT), which acylates the sn-1 position of glycerol-3-phosphate, acylglycerolphosphate acyltransferase (AGPAT), which acylates the sn-2 position, phosphatidic acid phosphohydrolase (PAPH), which forms diacylglycerol, the diacylglycerolcholine/ethanolamine phosphotransferases (CEPT) which form either phosphatidylcholine or phosphatidylethanolamine from diacylglycerol, and monoacylglycerol acyltransferase (MGAT) or diacylglycerol acyltransferase (DGAT) which can acylate diacylglycerol to form triacylglycerol. The formation of triacylated TG seed the formation of a nonpolar phase within the membrane domain, and this can grow and mature into either a lipid droplet or a VLDL particle. Other important phospholipid synthesis pathways exist at the ER but are not shown (Adapted from Fu et al, 2012).

plex is translocated to the Golgi. There, SREBP2 is proteolytically processed into an active transcription factor that induces transcription of HMG-CoA reductase, which is the rate-limiting enzyme in cholesterol biosynthesis and other sterol-regulated genes such as the LDL receptor (LDLR) [139]. Cholesterol clearance in the ER is managed by its conversion to oxysterols and eventually bile acids to be excreted into the bile [140], and the production of cholesterol esters that can be stored in lipid droplets [141].

The composition of the intracellular fatty acid pool is also regulated by the ER to meet the cellular requirements for complex lipids synthesis. The metabolic fate of fatty acids depends on a series of enzymatic reactions which rely on desaturases, elongases and β oxidation cycles. Desaturases and elongases occur mainly in the ER [142]. Fatty acids *de novo* synthesis is also partially controlled by fatty acid sensing mechanisms in the ER that regulate the SREBP1 transcription factor, via the same mechanism as described above (see SREBP2).

The ER contains two types of complex lipids – phospholipids and neutral lipids, as triglycerides and cholesterol esters. Phospholipids, due to its amphipatic nature, are an important component for membrane assembly in the ER and to vesicles that bud from the ER to the rest of the cell. Triglycerides and cholesterol esters, due to their hydrophobic nature, readily associate and form lipid droplets within the ER membrane [141]. The formation of lipid droplets protects the cells from the accumulation of excess of fatty acids and assures the integrity of the ER membrane.

However, while lipid droplets assemble as storage of intracellular lipids they require a specific increase in the synthesis of phosphatidylcholine (PC) at the ER, possibly altering the balance of the ER membrane [143]. In this way, lipid metabolism has an important role in maintaining ER membrane function, and even small perturbations to it can induce physiological changes that lead to ER stress. This is particularly important since recently, an emerging line of evidence indicates that ER stress in the liver is associated with subtle changes in the phospholipid composition of the ER [144].

All three major UPR pathways have been implicated in the regulation of cellular lipogenesis. During ER stress, activation of SREBPs occurs and plays a critical role in *de novo* lipid biosynthesis [145, 146].

In liver cells, XBP1s regulate the transcription of genes involved in fatty acid synthesis as *Scd-1* (stearoyl-CoA desaturase-1), *Acc2* (acetyl-CoA carboxylases 2) and *Dgat2* (diacyl glycerol acyl transferase 2). Thus, mice with a specific deletion of XBP1 in the liver have a severe impairment of *de novo* hepatic lipogenesis leading to reduced serum levels of triglycerides, cholesterol and free fatty acids [147]. In mice mammalian epithelium cells, deletion of another UPR sensor, PERK, inhibits the expression of genes involved in

fatty acid synthesis, such as *Fas* (fatty acid synthase), *Acl* (ATP citrate lyase) and *Scd-1*, resulting in a lower lipid content in the mammary gland and, consequently, a decrease in the free fatty acid pool present in the milk [148]. This phenotype appears to be dependent of SREBP1 activation via PERK.

Finally, investigation of liver lipogenesis when submitted to chemical ER stress induction with genetic mouse models indicated an impact of UPR on lipid metabolism [149]. When mice were exposed to tunicamycin (which disrupts N-glycosylation of newly synthesized proteins resulting in ER stress), liver tissue exhibited downregulation of genes as *Fas*, *Srebp1*, *PGC-1 α* (peroxisome proliferator-activated receptor-coactivator), *CEBP α* (CCAAT enhancer binding protein α), and *PPAR α* (peroxisome proliferator-activated receptor α); all are involved in lipid metabolic pathways. Livers from mice lacking ATF6, IRE1, p58IK or from an eIF2 α mutant strain, all presented an even higher impairment of metabolic gene expression with tunicamycin treatment when compared to livers from wild-type mice. On the contrary, livers from mice lacking CHOP were able to maintain metabolic gene expression under tunicamycin treatment, pointing to CHOP as a potential mediator of decreased gene expression under severe ER stress. Notably, tunicamycin is a non physiological ER stress inducer and is beyond what the liver experiences in metabolic fluctuations or even in most diseases. The data are, however, consistent with links between ER stress and liver lipogenesis. Independently of the genotype, tunicamycin challenge induces suppression of metabolic pathways and hepatic steatosis (i.e. abnormal retention of lipids). This seemingly paradoxical result (hepatic steatosis during defective liver lipogenesis) suggest a differential regulation of lipogenic genes during sustained ER stress as compared to physiological ER stress.

2.3.2 UPR in Glucose Metabolism

The liver has a central role in glucose metabolism owing to its capacity to take up and store glucose via glycogenesis, and synthesise and release glucose via glycogenolysis and gluconeogenesis [150]. Within the ER of hepatocytes, glucose-6-phosphatase (G6Pase) catalyzes the terminal step of hepatic glucose synthesis [151]. Thus, glucose metabolism is linked to the ER and all the three canonical branches of the UPR are committed to it. The first studies with genetic models of UPR deficiency reported defective gluconeogenesis in the livers of PERK-deficient mice, as well as in mice harbouring a homozygous mutation at the eIF2 α phosphorylation site (Ser51Ala) [117, 152]. The perturbed glucose metabolism seen in PERK-deficient mice and eIF2 α mutant mice was later linked to beta cell failure triggered by a defective ER [153]. A recent study further addressed the link between eIF2 α phosphorylation in the liver and glucose by enforced expression of the eIF2 α -specific

phosphatase, GADD34, in the liver. Transgenic mice for GADD34 displayed severe impairment in blood glucose when fasting as a consequence of the lower levels of glycogen storage in the liver, as well as impaired gluconeogenesis [154].

The ER is extremely sensitive to glucose availability and energy fluctuations, and both glucose synthesis and breakdown pathways are transcriptionally regulated during the UPR. Genes involved in glucose output and glycogen synthesis were identified as targets of XBP1 [155]. The ATF6 branch was linked to regulation of liver glucose through interaction with CRT2 (CREB-regulated transcription coactivator 2) [156]. Here, in response to ER stress, CRT2 binds to ATF6 and migrate to the nucleus where it binds XBP1 promoter. Interestingly, ATF6 may reduce hepatic glucose output by disrupting the CREB-CRT2 complex and, consequently, inhibiting CRT2 occupancy at gene promoters involved in gluconeogenesis.

Interestingly, the liver-specific UPR branch mediated by CREBH regulates hepatic gluconeogenesis by activating glucose genes in a CRT2 dependent manner [137]. Overall, the metabolic programmes linked to the ER are not simply pathways that are activated during ER stress. The metabolic status may be perceived by the ER in many different ways culminating in different adaptive responses. Such adaptive responses trigger additional stress signalling as well as inflammatory pathways and innate immunity, the subject of the next section.

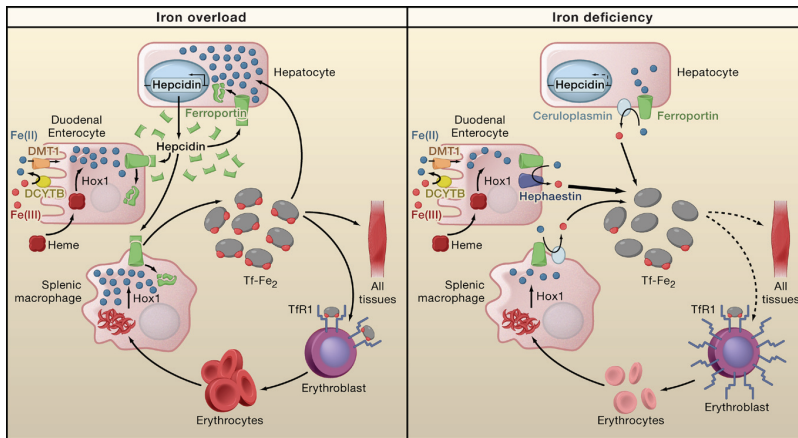


FIGURE 12

Hepcidin in systemic iron regulation.

Hepatocytes play a dual role in systemic iron metabolism - they are the major site of iron storage and they secrete the regulatory hormone hepcidin. Hepcidin, a 25-residue peptide hormone, is the central regulator of iron homeostasis. In iron overload conditions (left), high hepcidin levels inhibit iron export by binding to ferroportin, the only known iron exporter, and inducing its internalization, ubiquitination, and subsequent lysosomal degradation in hepatocytes and hepcidin target cells, as enterocytes and splenic macrophages. Iron egress is therefore inhibited decreasing its availability in circulation. In iron deficiency (right), hepcidin expression is low and iron is released by ferroportin into the circulation. Ferroportin-mediated iron export is coupled to the ferroxidase hephaestin (enterocytes) or the multicopper oxidase ceruloplasmin, a protein synthesized and secreted by the liver. Both facilitate iron extraction from the ferroportin channel and subsequent loading onto plasma transferrin. Iron import in enterocytes from the lumen of the duodenum is mediated by divalent metal transporter 1 (DMT1) at the apical membrane after DCYTB reduces Fe(III) to Fe(II). Hepatocytes also express DMT1. However, contrary to enterocytes DMT1 expression in the liver occurs in iron overload conditions. (Adapted from Hentze et al., 2010)

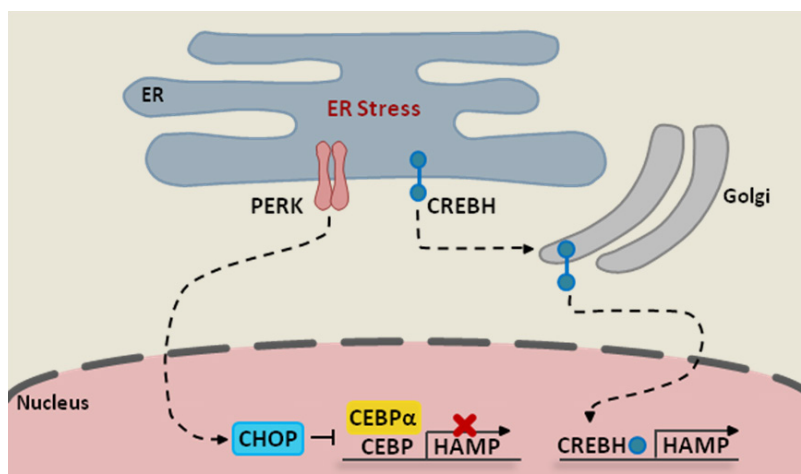


FIGURE 13
Regulation of Hepcidin Expression by UPR pathways.

Hepcidin expression in hepatocytes is regulated by multiple signals, which include systemic iron availability, hepatic iron stores, inflammatory/infectious states, anemia and hypoxia (Pigeon, et al., 2001; Nicolas et al., 2002). These different regulatory inputs are integrated transcriptionally, with iron stores and inflammation inducing its expression while the reverse occurs in the other conditions. Hence, multiple pathways coordinate hepcidin transcription. Recently, two different studies have uncovered a new pathway to hepcidin induction through UPR activation. Oliveira et al. showed that UPR activation in hepatoma cells with different ER stress inducers led to induction of hepcidin transcription. In this study hepcidin induction varied with ER stressors dose and time, with highest transcription induction with continued ER stress. The authors correlated the increase in hepcidin transcription with a decrease in the expression of the transcription factor CHOP. Previously reported, the C/EBPα isoform has been shown to activate hepcidin transcription (Courselaud et al., 2002). Additionally, C/EBPα can heterodimerize with other members of the same family, as CHOP (Ron and Habener, 1992). Oliveira et al. show that CHOP up-regulation depletes C/EBPα protein pool and hypothesized that, depending upon the level of ER stress, the initial induction of CHOP may bind to C/

EBPα at the HAMP promoter and repress hepcidin transcription. C/EBPα depletion by siRNA impaired hepcidin induction by UPR although did not abrogate it completely. This suggests that other mechanisms besides the CHOP-C/EBPα interaction are not the sole contributors of hepcidin regulation by the UPR. A study by Vecchi et al., suggests CREBH as a new regulator of hepcidin expression in the liver. The authors show that treatment of cultured hepatoma cells with multiple ER stressors induces hepcidin transcription, and confirmed the induction is maintained *in vivo*. In hepcidin promoter, Vecchi et al. show specific binding sites for CREBH and that ectopic expression of CREBH in nonhepatocyte cells induces hepcidin mRNA. Hepcidin induction by tunicamycin was impaired in CREBH knockout mice. Finally, immune activation of hepcidin induction with LPS was reduced and delayed in CREBH^{-/-} mice but not completely abrogated. These findings are in agreement with the involvement of multiple transcriptional pathways in hepcidin regulation.

2.3.3 UPR Crosstalk with Iron Metabolism

These studies demonstrate a link between ER stress and hepcidin production. The mechanisms proposed by both studies for hepcidin induction under ER stress are not incompatible and suggest that hepcidin is regulated differently according to different stress pathways.

2.4 UPR and Inflammation

A growing body of evidence has demonstrated clear links between UPR pathways and the regulation of inflammation and innate immunity (FIGURE 14). During ER stress, IRE1 α triggers activation of JNK which in turn induces the expression of inflammatory genes by phosphorylating the transcription factor activator protein 1 (AP1) [104]. NF- κ B is a key transcriptional regulator that has a central role in inflammation [157]. Through multiple and distinct mechanisms, both the IRE1 α and PERK branches of the UPR are able to activate NF- κ B-IKK pathway [107, 158]. The same pathway has been recently linked to the ATF6 branch of the UPR, suggesting that a specific inflammatory trigger can be activated through different UPR branches [159]. Experimental activation of UPR in cellular systems has been reported to increase the expression of proinflammatory molecules such as interleukin 8 (IL-8), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor- α (TNF- α) [160]. In the liver, in response to ER stress, CREBH activation induces the expression of serum amyloid P-component (SAP) and C-reactive protein (CRP), conferring to CREBH a role as a regulator of the acute phase response [135].

Chronic ER stress and UPR activation can lead to oxidative stress, causing a toxic accumulation of reactive oxygen species (ROS) within the cell [161]. This occurs due to the UPR-induced upregulation of protein chaperones involved in disulfide bond formation in the ER lumen. The formation of disulphide bonds in proteins in the ER is driven by the enzymes PDI and ERO1 (ER oxidoreductin 1). These enzymes use oxidation/reduction reactions with the molecular oxygen as the terminal electron recipient, leading to the production of ROS [162]. Hence, the UPR has evolved mechanisms to limit the accumulation of ROS during ER stress via PERK-mediated activation of an antioxidant program, through the transcription factor Nrf2. Once activated, Nrf2 induces transcription of oxidant-detoxifying enzymes, hence neutralizing the toxic species. However, prolonged UPR activation may still result in increased ROS levels, which may elicit inflammatory responses, proving to be another link between ER stress and inflammation.

Recently, ER stress was shown to boost innate immunity and inflammatory responses. For example, macrophages treated with pharmacological agents that induce ER stress display an enhanced response to TLR4 and TLR2 acti-

ER stress is linked to inflammation through the activation of the JUN N-terminal kinase (JNK) and the I κ B kinase (IKK)–nuclear factor- κ B (NF κ B) pathways, and through cyclic-AMP-responsive-element-binding protein H (CREBH) activation by the UPR. These pathways result in the induction of an inflammatory response. Activation of JNK can also serine phosphorylate insulin receptor substrate 1 (IRS1), resulting in altered metabolic responses. Key organelles for cellular metabolism, such as the ER, Golgi, mitochondria and peroxisomes (not shown) are connected through an endomembrane network, which provides functional continuity between organelles that can therefore share functional information in the form of

lipids and proteins at specific contact sites. This functional and molecular integration between the organelles can mediate the spread of stress from one organelle to the other, resulting in exacerbation of inflammation and cytotoxicity during chronic metabolic stress conditions such as obesity and dyslipidaemia. AP1, activator protein 1; ATF6f, ATF6 fragment; eIF2 α , eukaryotic translation initiation factor 2 α ; I κ B, inhibitor of NF κ B; XBP1s, spliced X-box binding protein 1 (Adapted from Hotamisligil and Erbay, 2008).

vation [163, 164] being XBP1 required for the maximal induction of several genes, including IL-6, by membrane toll like receptors (TLRs) [163]. This suggested that components of ER-signalling pathways are intimately connected to innate immune responses. In the study by Martinon *et al*, the authors reported that in the absence of detectable ER stress, TLR4 and TLR2 specifically promoted IRE1 α phosphorylation and activation of XBP1. However, IRE1 α activation did not activate other ER-signalling pathways. TLR-activated XBP1 was required for optimal and sustained production of proinflammatory cytokines in macrophages. The role of XBP1 in innate immunity was maintained *in vivo*, where XBP1 deficient mice exhibited increased bacterial burden when infected with the TLR2-activating bacteria *Francisella tularensis*. These observations demonstrate that specific branches of ER signalling pathways are directly used by innate immune pathways in the absence of ER stress. In *Caenorhabditis elegans*, XBP1 and ATF6 are also activated upon infection with pore-forming toxin bacteria, which act as a defence against these pathogens [165].

In a different model, the activation of the innate immune kinase PMK-1 in *C. elegans* infected with *Pseudomonas aeruginosa* induced IRE1 and XBP1 activation. Here, XBP1 loss of function impairs the infected worms viability, suggesting that XBP1 suppresses the deleterious effects of PMK-1 activation during the immune response [166].

These studies suggest that while ER stress activation may enhance innate immune responses, infections and innate immune pathways may redirect the ER stress response to enhance ER signalling pathways that potentiate inflammation, while down-regulating other pathways. In support of the aforementioned hypothesis it has been reported that the ATF4-CHOP branch of the UPR can be specifically inhibited by TLR signalling [167], since in macrophages TLR signalling inhibited ATF6 and PERK activation [163].

Thus, by integrating several pathways, the ER is a hub of cellular and organism homeostasis and so modulation of its activities might contribute to disease. Indeed, several metabolic and infectious diseases are intimately connected with the UPR. Metabolic syndromes, as obesity and type 2 diabetes and chronic viral hepatitis B and C are just a few examples of diseases associated with UPR activation.

3 STUDY OUTLINE

Plasmodium, the agent that causes malaria, is an obligate intracellular pathogen. Thus, while this feature can allow parasites to evade some host immune responses it also suggests the exploitation of host cell resources to support *Plasmodium* developmental needs. In the liver, *Plasmodium* parasites establish, within hepatocytes, one of the most remarkable replication processes, hinting that complex interactions between the two living cells, hepatocyte and parasite, must occur to culminate in a successful infection. Indeed, as previously mentioned, during intrahepatic development *Plasmodium* parasites induce significant alterations to the hepatocytes metabolic signature, such as lipid and glucose metabolism [61].

In highly metabolically active cells, like hepatocytes, the endoplasmic reticulum (ER) is essential in the maintenance of cellular homeostasis; several infectious and metabolic diseases are characterized by the activation of signalling pathways steaming from the ER. The Unfolded Protein Response (UPR) is regulated by endoplasmic reticulum sensors and, while first described as a stress response, its functions are increasingly being recognized to impact on cellular homeostasis. As such, UPR activation has been associated with diseases such as type 2 diabetes, obesity, atherosclerosis, neurodegenerative diseases but also infections with intracellular pathogens, such as viruses. While UPR is well documented in viral infections, its role during infections with eukaryotic pathogens is unknown.

In this Doctoral Thesis, we hypothesized that *Plasmodium* development within hepatocytes might impact on ER function and modulate UPR signalling pathways. By engaging the host cell resources to its own development, the alterations induced by *Plasmodium* to the host cell metabolic repertoire must be integrated within ER functions through the UPR sensors. Thus, our overall goal was to understand how UPR pathways can impact on *Plasmodium* infection. To address this question, we first examined two studies comparing infected versus uninfected cells at the transcriptional and proteomic levels, in order to establish whether changes in the endoplasmic reticulum transcriptome/proteome could be found, including an ER stress signature. We further established functional assays with UPR activation strategies and genetic models of UPR disrupted pathways and assessed its impact on *Plasmodium* liver infection.

Understanding the nuances of the interactions of *Plasmodium* with UPR pathways in the liver infection context is crucial to acknowledge that, although asymptomatic, the malaria liver stage is far from being silent and our data suggests that the ER plays an important role in malaria liver pathogenesis.

All the work presented in this thesis was performed by Patrícia Inácio with the exception of the proteomics study which was performed as a collaboration between our laboratory and the laboratory of Mathias Mann, Max Planck Institute of Biochemistry in Munich, Germany.

RESULTS

CHAPTER TWO

4 PROTEOMICS, MICROARRAYS AND QRT-PCR VALIDATION

Plasmodium infection induces expression of ER stress markers

Hepatocytes uniqueness is, as previously mentioned, intimately connected to its enrichment in endoplasmic reticulum (ER). We confirmed the abundance of ER in hepatocytes by staining for calnexin, a molecular chaperone and ER marker, and observed that the vacuole where *Plasmodium berghei* develops is closely surrounded by this organelle (FIGURE 15), although no association with the host ER was observed, as previously described [168]. To determine the impact of *Plasmodium berghei* infection on the proteome landscape of hepatocytes and, more specifically, on the ER, we performed an analysis of a comparative semi-quantitative proteomic study on *P. berghei* infected and uninfected cells performed in collaboration between our laboratory and the laboratory of Prof. Mathias Mann (Max Plank Institute of Biochemistry, Munich) (Zuzarte-Luís, *unpublished*). For this study, and owing to the extremely low sporozoite infectivity [69], cells from the human hepatoma cell line Huh7 were infected with GFP-expressing *P. berghei* ANKA sporozoites [169] and infected cells were isolated by Fluorescence Activated Cell Sorting (FACS), 12 hours post-infection (pi), as previously reported [61]. This allowed the enrichment and purification of the infected cell population. The total number of proteins identified was 1988, 687 of which were differentially expressed (DE) in *P. berghei* infected cells. The proteins identified as DE were then analysed in the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov/>) ([170],[171]). Due to our interest in the ER, we determined the subcellular localization of the upregulated DE proteins with the 'Cellular Component' subset of Gene Ontology included in the DAVID System. Interestingly, despite no ER fractionation was performed (which would allow to collect/concentrate ER proteins), we found an enrichment of proteins of the endoplasmic reticulum and nucleus-endoplasmic reticulum network, as

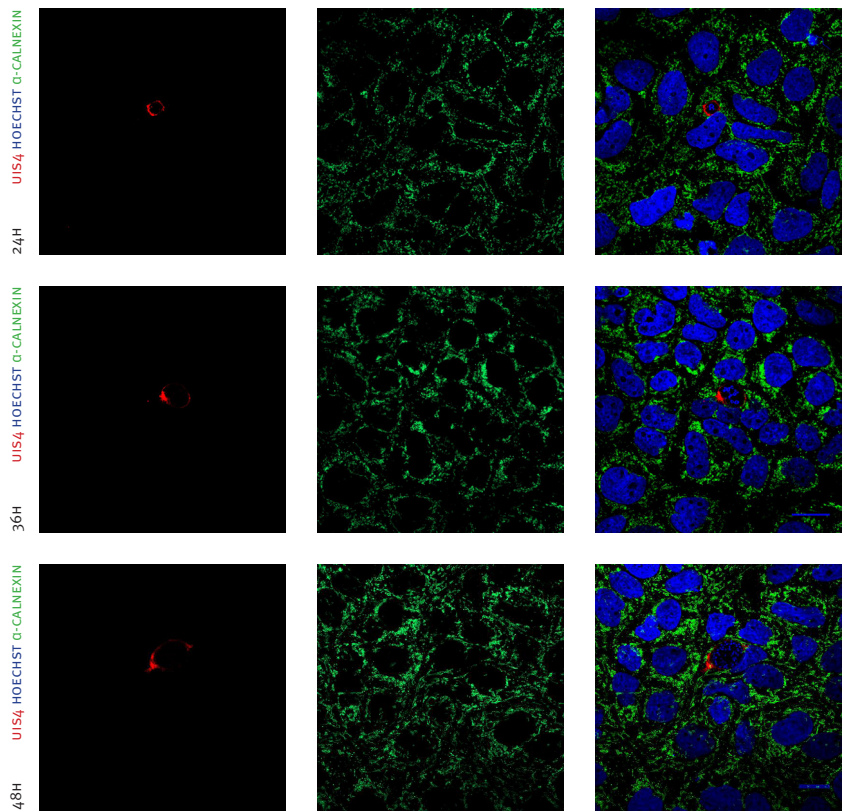


FIGURE 15

Host cell calnexin distribution in hepatoma cells infected with *Plasmodium berghei* at 24, 36 and 48 hours of infection.

Hepa1-6 cells were infected with *Plasmodium berghei* sporozoites and infection was stopped at the mentioned time point after infection. Host endoplasmic reticulum was stained with anti-calnexin antibody (green), *Plasmodium* was stained with anti-UIS4 antibody (red) and nucleus with hoechst. Scale bar 22 μ m.

well as mitochondrial proteins (not shown). In the MA plot we highlighted the DE proteins identified as endoplasmic reticulum proteins by gene ontology (GO) (FIGURE 16). We plotted the normalized log intensities of the 60 DE endoplasmic reticulum proteins (FIGURE 17) where a clear cluster was identified as upregulated with infection. Among those, gene ontology analysis identified an enrichment of proteins involved in the process of protein folding, as demonstrated by the induced expression of calnexin (*Canx*) and calreticulin (*Calr*), which play important roles in the ER quality control apparatus through retention of incorrectly folded proteins ([172],[173]); the endoplasmic reticulum resident protein 29 (*Erp29*) [174] and ERp44 (*Erp44*) [175], both important to the correct protein folding, together with ERp19 (*Txndc12*) and ERp46 (*Txndc5*), members of the thioredoxin family of ER proteins, which are highly expressed in the liver [176]; and protein-modifying enzymes (the upregulation of which facilitates protein folding), as dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 and 2 (*Rpn1/Rpn2*) and mannosyl-oligosaccharide glucosidase (*Mogs*) [177]. On the other hand, proteins involved in the ER-associated protein degradation (ERAD) were also overrepresented, as endoplasmic reticulum chaperone (*Hsp90b1*), both components of the ERLIN1/ERLIN2 complex, together with the UBX domain-containing protein 4 (*Ubxn4*, also known as *Ubx2*) [178]. The upregulation of the protein folding capacity, together with ERAD activation is consistent with the induction of the unfolded protein response (UPR), in response to ER stress. In fact, ERp44 and UBX domain-containing protein 4 are induced, at the protein level, with ER stress ([175],[179]). Moreover, the major ER stress marker, the 78 kDa glucose-regulated protein, mostly known as BiP (*Hspa5*), together with protein disulfide-isomerase, also known as PDI (*P4hb*) [180] were clearly upregulated with infection. Altogether, our data analysis on ER proteins show that *Plasmodium* infection induces the expression of proteins that represent a clear signature of UPR activation in response to ER stress (FIGURE 17).

Additionally, we re-analysed the transcriptome of infected mouse Hepa 1.6 cells at different time-points from a genome-wide microarray assay recently performed in our laboratory [61]. Once again, we identified transcriptional upregulation of several key ER stress markers like *Atf4* and *Chop* (also known as *Ddit3*), XBP1 target gene *Dnajb9* (although no XBP1 induction was observed), *Atf6* together with *Atf3*, *Cebpb*, *Herpud1* and *Trib3*, already after 6 hours of *P. berghei* infection (FIGURE 18).

To independently validate the microarray results, we analysed the expression of 4 transcripts by quantitative RT-PCR (qRT-PCR), selected for their high induction levels and well established role in ER stress [181]. Owing to *Plasmodium* low infectivity [69], Hepa 1.6 cells were infected with GFP-ex-

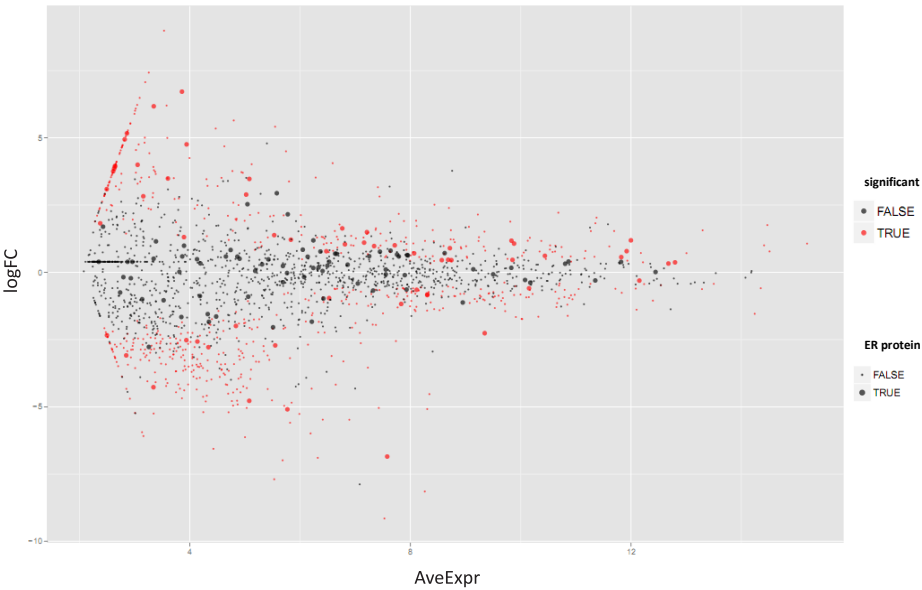


FIGURE 16
Highlighting endoplasmic reticulum DE
proteins at 12 hours pi MA plot.
Endoplasmic reticulum proteins identified
by gene ontology (GO_0044432) in 12 hours
MA plot.

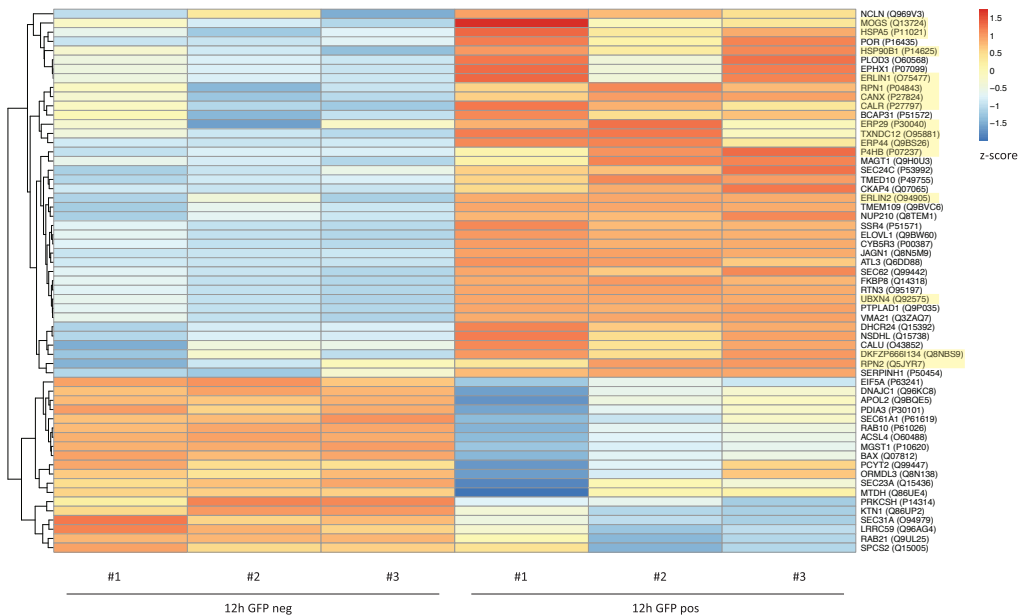


FIGURE 17
Host endoplasmic reticulum proteins identified as DE proteins at 12 hours pi.
Heatmap of DE proteins identified as endoplasmic reticulum proteins by gene ontology (GO) (GO_0044432). Each row of the plot is a protein and was coloured according to row-normalized log-intensity (z-core) with red meaning up-regulation and blue meaning down-regulation. Each row is identified with gene name and UniProt Accession number. Each column represents a replicate. Highlighted in yellow are all the proteins mentioned in the text.

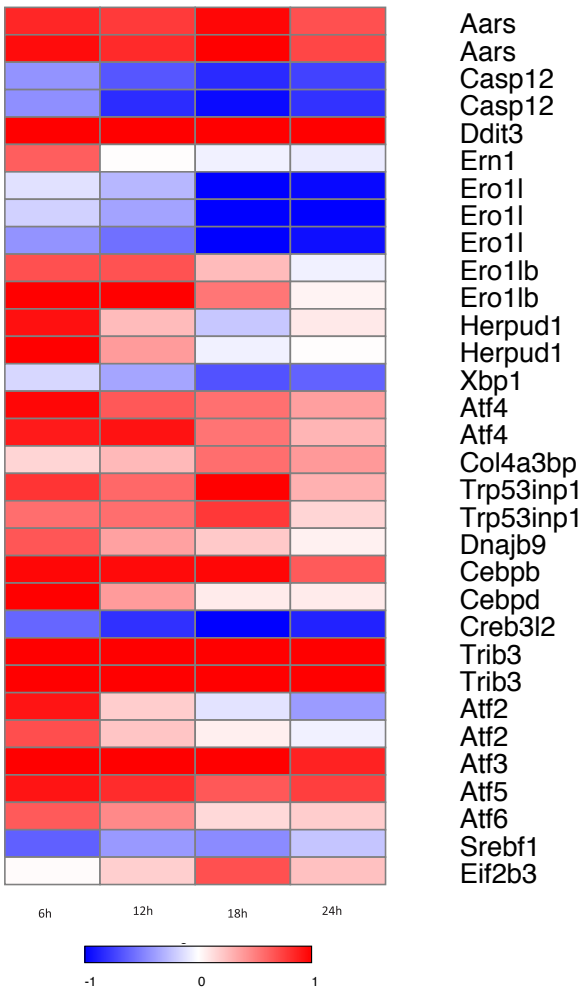


FIGURE 18
Host cell transcriptional profiling reveals activation and modulation of UPR components during malaria liver stage infection *in vitro* (re-analyzed from Albuquerque et al. 2009).
Heatmap of DE transcripts involved in ER stress/UPR pathways in an *in vitro* liver stage infection at the mentioned time points of infection. Each row of the plot is a gene and was coloured according to the log₂ ratio of expression, with red meaning up-regulation and blue meaning down-regulation.

pressing *P. berghei* ANKA sporozoites [169] and purified by FACS at 6 and 24 hours pi. Hepa 1.6 GFP-negative cells, from each time point, were used as control for basal mRNA expression. At both time-points of infection, *Plasmodium* induces the expression of ER stress markers as *Atf4*, *Chop*, *Atf3* and *Herpud1* (FIGURE 19), in accordance with the microarray results.

In summary, different but complementary approaches (analysis of proteomics/transcriptomics analysis and qRT-PCR) show that hepatocyte infection by *Plasmodium* sporozoites induces ER stress with concomitant UPR activation.

4.1 The role of UPR activation in *Plasmodium* liver infection

***In vivo* UPR activation results in increased numbers of exoerythrocytic forms (EEFs)**

In highly metabolic organs such as the liver, the UPR is a central regulator of metabolism. To determine the impact of UPR activation on infection we pre-stimulated mice with tunicamycin (TM), a potent pharmacological ER-stress inducer widely used in ER stress associated research [181]. Eight hours later, TM-treated and non-treated mice were infected with luciferase-expressing *P. berghei* sporozoites. Sporozoite delivery was carried out by intravenous injection (iv) or via mosquito bite (12 mosquitoes per mouse for 30 minutes). Light emission from the stably integrated luciferase gene was used to quantify parasite liver load [182]. At 42 hours pi we detected a highly significant increase in parasite load in ER stress induced livers (FIGURE 20A, B). ER stress was assessed by XBP1 splicing assay (FIGURE 20C). *P. berghei* delivered by mosquito bite to mice treated with TM equally resulted in a marked increase on infection (FIGURE 21). The observed increase in parasite load can result from (1) a higher number of parasites that manage to reach and establish a successful liver infection or (2) an increase in parasite replication while the number of infected cells remains constant. To determine the cause of the infection increase we performed liver sections from TM and control untreated mice and quantified parasite numbers and growth by fluorescent microscopy for the GFP-expressing parasites. Microscopic examination revealed that the increase in infection (FIGURE 22A) is due to higher exoerythrocytic form (EEFs) numbers rather than EEF size (FIGURE 22B, C).

We then investigated when during liver stage infection EEF numbers increase can be observed. After ER stress induction with TM we infected mice and quantified infection by qRT-PCR in 3 groups of mice sacrificed at 14, 24 and 44 hours after sporozoite delivery by iv injection. Here, qRT-PCR of the 18S ribosomal RNA gene was chosen instead of the luciferase assay due to its higher sensitivity, particularly during early stages of parasite infection. The increase in parasite numbers can already be detected at 24 hours after

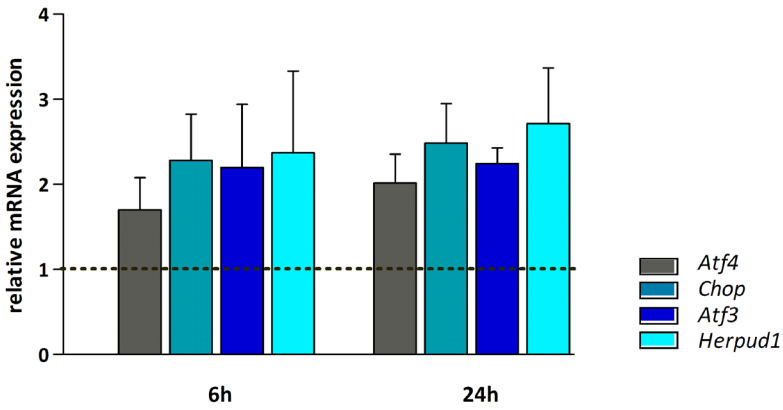


FIGURE 19
Real time PCR analysis of mRNA expression of ER stress markers in infected sorted cells.
Real-time PCR analysis of *Atf4*, *Chop*, *Atf3* e *Herpud1* mRNA in sorted Hepa 1.6 cells infected with *P. berghei* at 6 hours and 24 hours post-infection. Normalization to its respective time-point salivary gland control (---) Results are expressed as means $\pm$ s.m. (n=3 independent experiments).

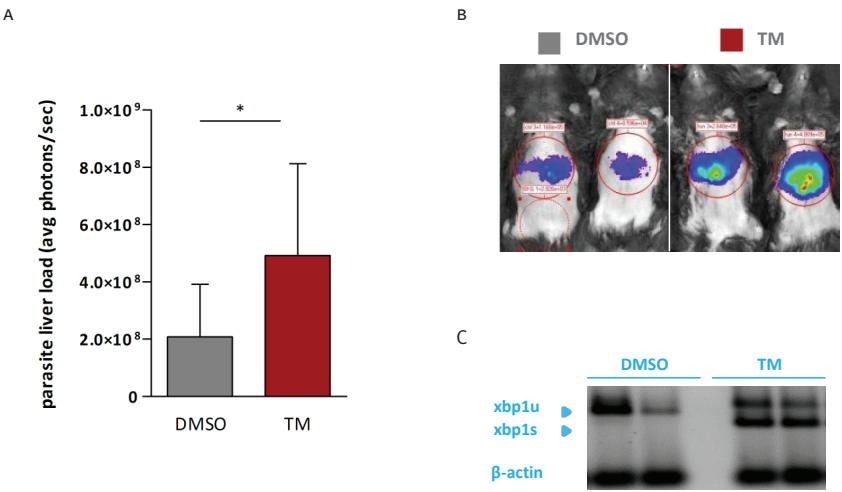


FIGURE 20
Mice pre-treated with tunicamycin (TM) show increased liver infection.
Plasmodium berghei (Pb) liver load measurement on mice treated with DMSO (control) or tunicamycin (TM) for 8 hours prior to sporozoite delivery by i.v. injection. Average radiance at 42 hours after luciferase-Pb injection (A) Representative live imaging of luciferase-Pb infection on DMSO and TM treated mice at 42 hours of infection (B) * $p < 0.05$, t test. Results are expressed as means $\pm$ s.d. (n=5 mice per group) (n = 2 independent experiments) TM at 1 μ g per g body weight. RT-PCR analysis of XBP1 maturation with TM treatment (U – unspliced S-spliced) (C).

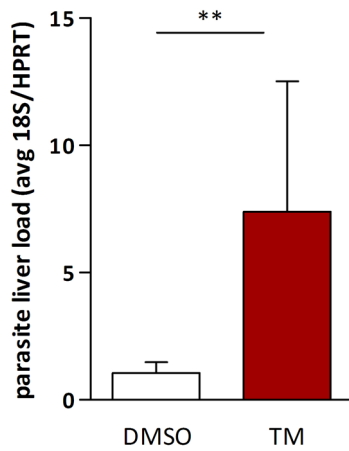


FIGURE 21

Mosquito bite delivery of sporozoites to mice treated with TM exhibit a significant increase on infection.

Plasmodium berghei (Pb) liver load measurement on mice treated with DMSO (control) or tunicamycin (TM) for 8 hours prior to sporozoite delivery by mosquito bite. Infection measured at 42 hours by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (Hprt) expression. ** $p < 0.01$, t test Results are expressed as means $\pm$ s.d. (n=5 mice per group) (n=2 independent experiments). Tunicamycin at 1 ug per g body weight.

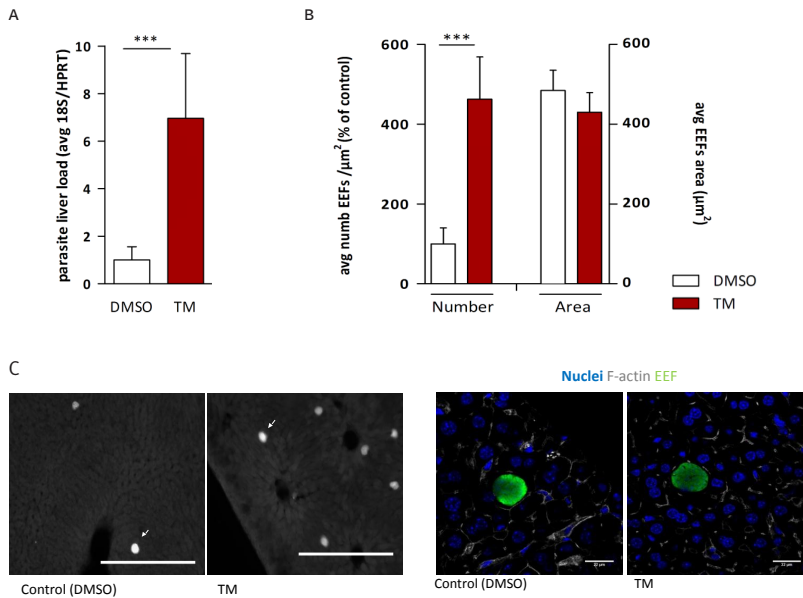


FIGURE 22

***In vivo* treatment with TM increases sporozoite EEF number but not EEF size.**

Plasmodium berghei (Pb) liver load measurement on mice treated with DMSO (control) or tunicamycin (TM) for 8 hours. Infection measured at 42 hours by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (Hprt) *** $p < 0.001$, t test. Results are expressed as means $\pm$ s.d. (A) Fluorescent microscopy quantification of EEF number and development (EEF area) on DMSO and TM liver sections at 42 hours after sporozoite injection ** $p < 0.01$, t test. (n=3 independent experiments) Results are expressed as means $\pm$ s.d. (B) Representative fluorescent microscopy images on EEF numbers and area on liver sections quantified in (B) (C)

sporozoite infection and is maintained at 44 hours pi (FIGURE 23). Together these results clearly show that TM-induced ER stress leads to a very significant increase in infected hepatocyte numbers, which is detectable at early stages of infection.

UPR activation in primary hepatocytes has no effect on infection outcome

We next tested whether UPR activation with TM results in increased infection rates in primary hepatocyte cultures similar to what we observed in the *in vivo* experiments. Treatment of mouse primary hepatocytes with varying TM concentrations (0.5 and 10 ug/ml) had no effect on infection rates (FIGURE 24A). ER stress activation was assessed by XBP1 splicing assay (FIGURE 24B). These data suggest that the observed TM effect *in vivo* is dependent on signalling events that are absent or under-represented in *ex vivo* models, as metabolic or inflammatory pathways.

UPR activation does not inhibit recruitment of inflammatory cells

The hallmark of inflammation is the recruitment of inflammatory cells to the site of infection which relies on signalling events, controlled by chemokines, between cells under attack and their environment ([183],[184]). The recruitment of such cells can, however, be inhibited to promote pathogen survival. A previous study, for example, reported that heme oxygenase-1 (HO-1) is induced during malaria liver stage infection by several *Plasmodium* species. HO-1 levels correlated with parasite numbers as it protects developing parasites from an inflammatory response by inhibiting the induction of cytokines and recruitment of inflammatory cells to the liver [185]. Therefore, we sought to determine whether increased parasite numbers following TM treatment were correlated to increased HO-1 levels and inhibition of the inflammatory response. Using qRT-PCR, we quantified both HO-1 and lineage-specific cell markers. As shown in FIGURE 25A, livers treated with TM show increased levels of HO-1 (2.5 fold) while we found no decrease in inflammatory cells. In fact, macrophages and neutrophils have an even higher liver penetration in TM treated livers at 42 hours pi (FIGURE 25B). Therefore, although HO-1 levels are high, inhibition of an inflammatory response is not likely the cause for the observed increase in parasite numbers after treatment with TM. We further explored this aspect and since the increase in infection in TM treated livers is already observed early during infection, we quantified the same cells at 24 hours pi. The recruitment of inflammatory cells does not occur early in infection, since we detect no differences between sporozoites and salivary glands infected mice, and TM treatment has no effect on the inflammatory cell burden in the liver at 24 hours pi (FIGURE 26). Thus, the phenotype observed with UPR activation is likely dependent

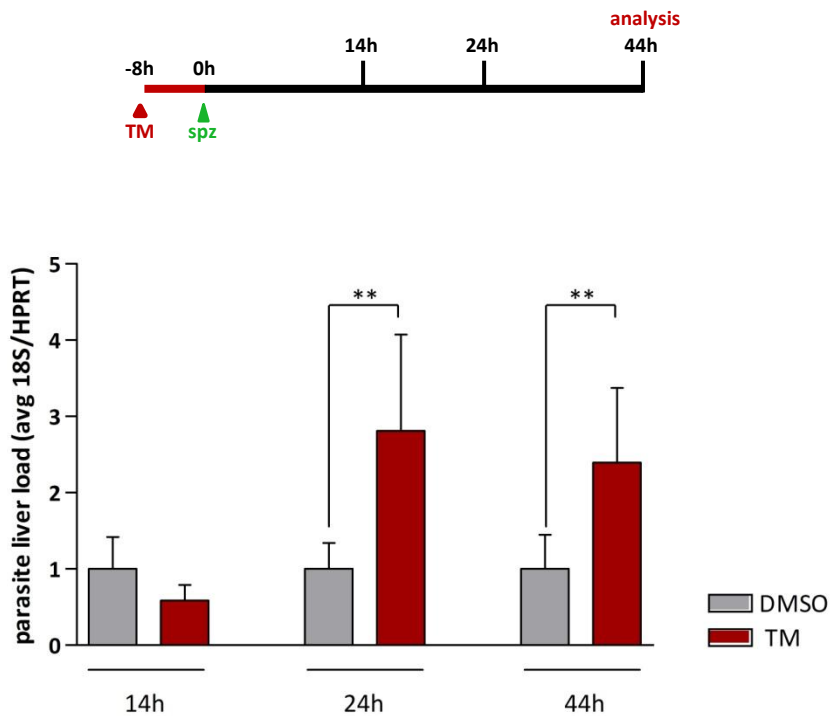


FIGURE 23
EEF numbers increase in TM treated mice occurs between 14 and 24 hours of infection.

Plasmodium berghei (Pb) liver load quantification at 14, 24 and 44 hours after i.v. sporozoite injection on mice treated with DMSO (control) or tunicamycin (TM) for 8 hours. Infection measured by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hprt) ** p < 0,01, t test. Results are expressed as means ± s.d. (n=2 independent experiments).

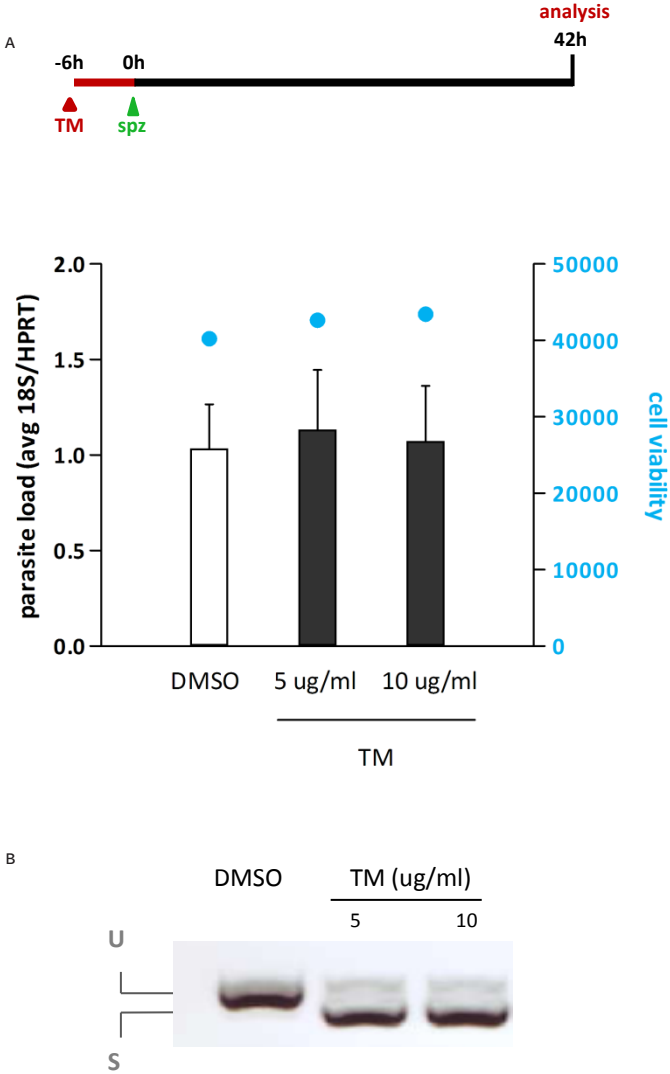


FIGURE 24
Incubation of primary hepatocytes with TM has no effect on infection outcome.
Mouse primary hepatocytes treated for 6 hours, prior to infection, with tunicamycin (TM, 5ug/ml and 10ug/ml). Infection measured at 42 hours by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hppt) expression. Cell viability was measured by Alamar Blue assay (fluorescence intensity). Results are expressed as means $\pm$ s.d. (n = 3 independent experiments) (A). RT-PCR analysis of XBP1 maturation (U – unspliced S-spliced) (B).

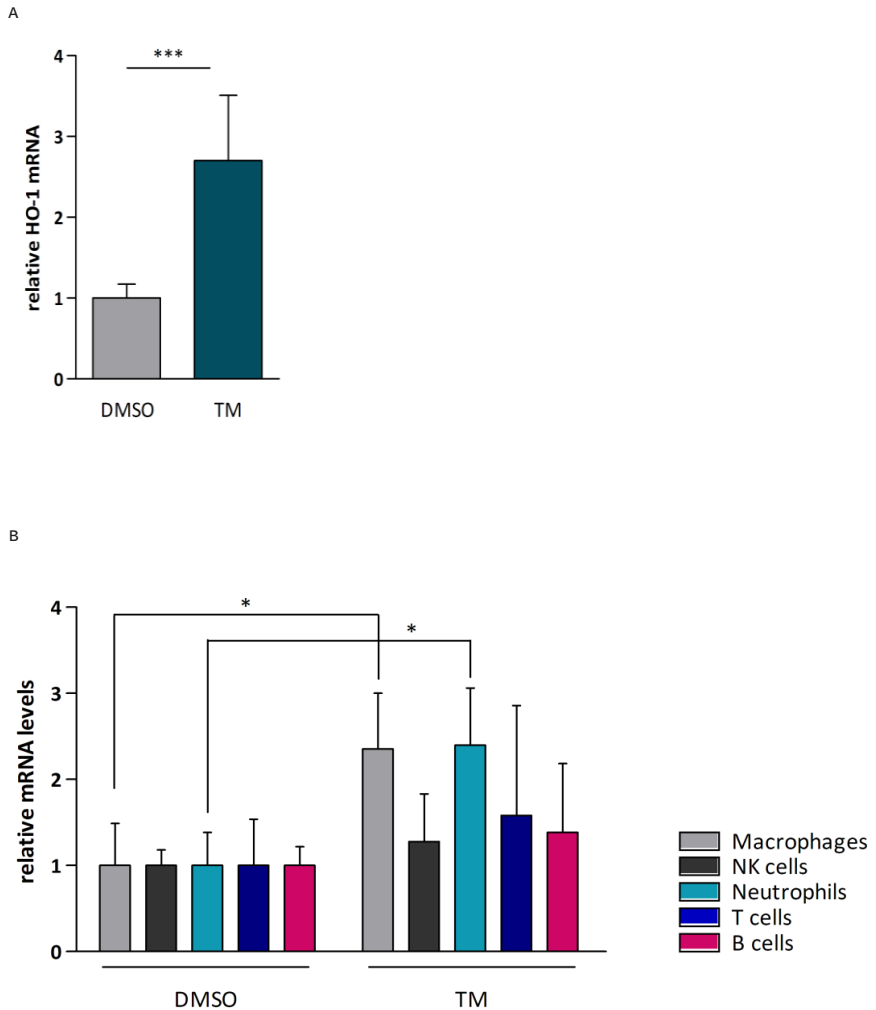


FIGURE 25

TM treated livers show no impairment in inflammatory cells at late liver infection.

Quantification of heme-oxygenase 1 (HO-1) (A) and inflammatory cells (B) in livers pre-treated for 8 hours with TM or DMSO (control). The mRNA levels of lineage specific-markers (*Mgl2* – macrophages, *Klrb1* – NK cells, *Ncf2* – neutrophils, *CD3* – T cells, *CD19* – B cells) were measured by qRT-PCR analysis and normalized to HPRT in liver extracts taken 42h after sporozoite i.v. injection. Results are plotted as fold change over the respective DMSO controls. * $p < 0,05$, t test. Results are expressed as means $\pm$ s.d. (n=4 per group).

on liver resident cells. Since the liver is composed in its majority by hepatocytes, this result supports the hypothesis that UPR and infection might be linked through metabolic pathways. However, the increase in parasite burden and the fact that it happens early in infection suggests that a fast-acting response, such as innate immunity, can be impaired in TM treated mice.

TM treatment impairs ISG mRNA induction upon sporozoite infection

Innate immunity induces a type-I interferon (IFN) immune response, which is particularly important against intracellular pathogens [186] (for a detailed mode of action of type I IFN response see FIGURE 27). Interestingly, recent work from our lab suggests a type I IFN response in malaria liver stage (Liehl *et al.*, unpublished). UPR pathways and innate immunity have been shown to regulate each other. While UPR pathways can activate and enhance inflammatory responses ([187],[188]), UPR activation can also inhibit innate immune responses against intracellular pathogens ([189], [190]). Thus, we sought to investigate whether the TM-induced increase on infection is linked to a type-I interferon response. We measured IFN- β mRNA expression together with 10 different type-I interferon stimulated genes (ISGs) in mouse livers after 4 different individual treatments – 8 hours DMSO, 8 hours TM, 44 hours sporozoite infection and the dual effect of 8 hours TM with 44 hours sporozoite infection. Sporozoite infection alone significantly induces the expression of IFN- β mRNA together with all ISGs. However, mice with both TM treatment and sporozoite infection exhibit a decrease in ISGs induction when compared with sporozoite alone (FIGURE 28A, B). In fact, TM treatment for 8 hours without infection already decreases the basal level of most ISGs mRNA expression tested (FIGURE 28B). Sporozoite induction and TM impairment of ISGs is also observed at the protein level, as illustrated for DAI (FIGURE 29). These data suggests that *in vivo* treatment with TM impairs a type-I interferon immune response induced upon sporozoite liver infection.

To further confirm the decrease in ISGs induction with TM treatment, we treated mice with the chemotherapeutic agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a potent inducer of type I IFNs [191], prior to TM treatment. Similar to the results obtained with sporozoite infection, treatment with DMXAA substantially induced the expression of the tested ISGs, while TM treatment decreases its induction (FIGURE 30).

Infection increase is partially dependent on type I interferon response

The experiments presented above show that activation of a type I IFN response in *Plasmodium* liver infection is disrupted in mice pre-treated with TM. To test if the infection increase depends on an impaired type I IFN response we used

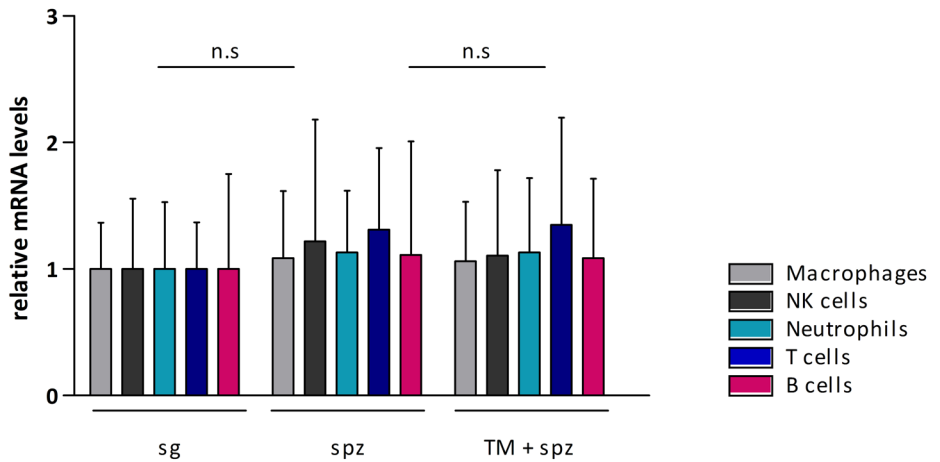


FIGURE 26

The recruitment of inflammatory cells to the liver does not occur at 24h after spz injection.

Quantification of inflammatory cells in DMSO pretreated mice injected with salivary glands (sg) or spz and in TM pretreated mice for 8h injected spz. The mRNA levels of lineage specific-markers (*Mgl2* – macrophages, *Klrb1* – NK cells, *Ncf2* – neutrophils, *CD3* – T cells, *CD19* – B cells) were measured by qRT-PCR analysis and normalized to HPRT in liver extracts taken 24h after sporozoite i.v. injection. Results are plotted as fold change over the respective uninfected DMSO control mice. Results are expressed as means $\pm$ s.d. (n=5 per group) (3 independent experiments).

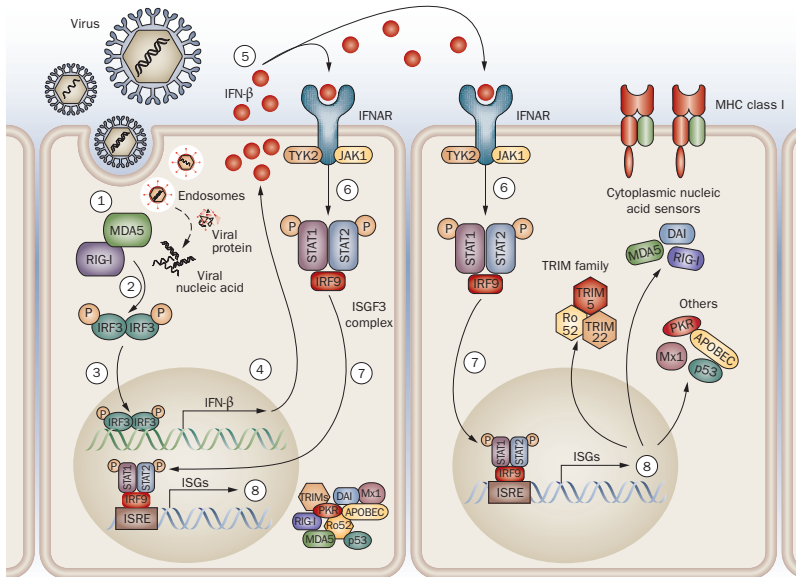


FIGURE 27

Type I interferon (IFN) mode of action in response to intracellular pathogens (e.g. viruses).

Viral particles are endocytosed (1) and the release of viral nucleic acids is triggered in the cytoplasm (2) where it's recognized by cytosolic nucleic-acid-sensing receptors (such as RIG-I, MDA5 and DAI) which induces phosphorylation of IRF3. IRF3 once phosphorylated dimerizes and travels to the nucleus (3) where in association with additional transcription factors induce transcription, primarily at the IFN β locus (4). IFN- β is produced and secreted from the infected cell (5). Once secreted, IFN- β acts in an autocrine and paracrine manner and binds the receptor IFNAR expressed in infected and neighboring cells. IFNAR induces the formation of phosphorylated STAT1/STAT2 heterodimers (6) which by associating to IRF9 form the ISGF3 complex (7). ISGF3 complex translocates to the nucleus inducing the transcription of hundreds of interferon stimulated genes (ISGs) (8). (Adapted from Hall and Rosen, 2010).

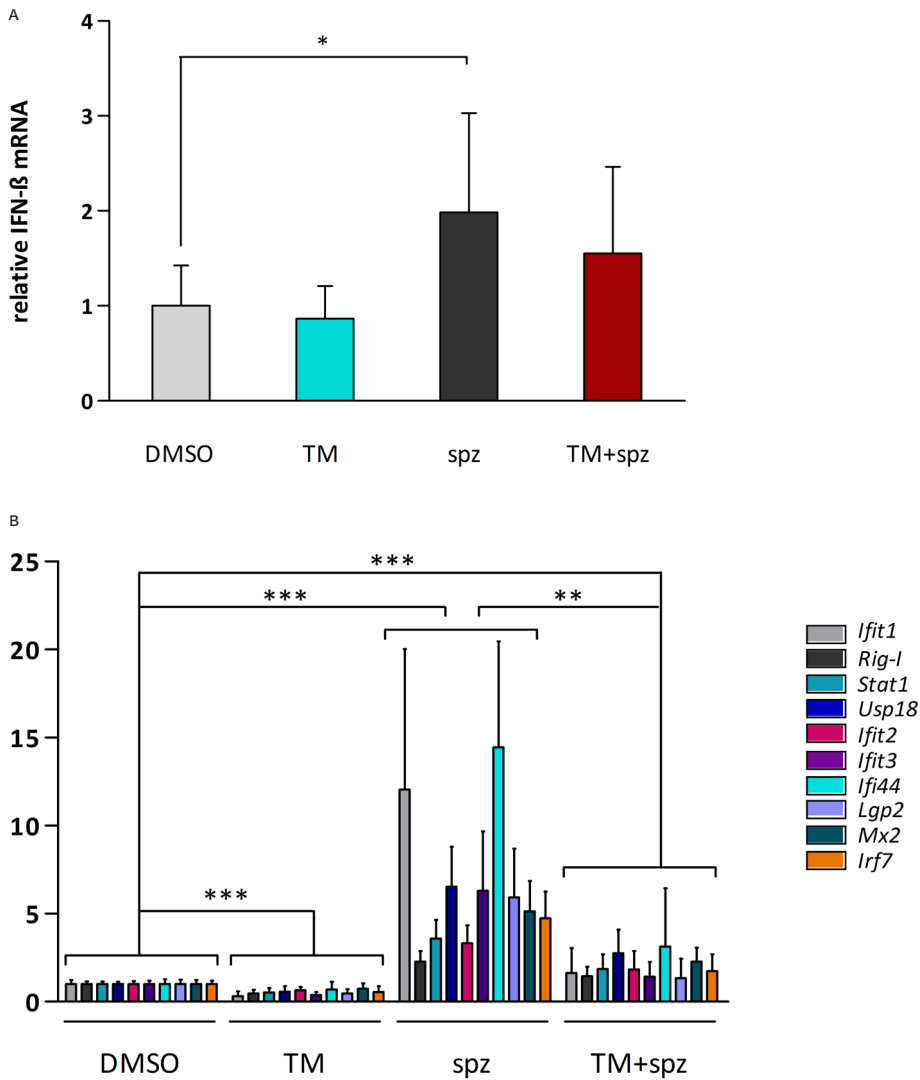


FIGURE 28

Inhibition of interferon type-I response in TM treated mice.

Real-time PCR analysis of Interferon Beta (IFN- β) (A) and Interferon Stimulated Genes (ISGs) (*Ifit1*, *RIG-I*, *Stat1*, *USP18*, *Ifit2*, *Ifit3*, *Ifi44*, *Lgp2*, *Mx2* and *Irf7*) (B) mRNA in mice livers treated for 8h with DMSO and TM, liver extracts taken 44h after sporozoite i.v. injection and liver extracts of mice treated with TM (8h) and taken 44h after sporozoite i.v. injection (n=5 per group) ** p < 0,01, *** p < 0,001, t test. Results are expressed as means $\pm$ s.d. (n=2 independent experiments).

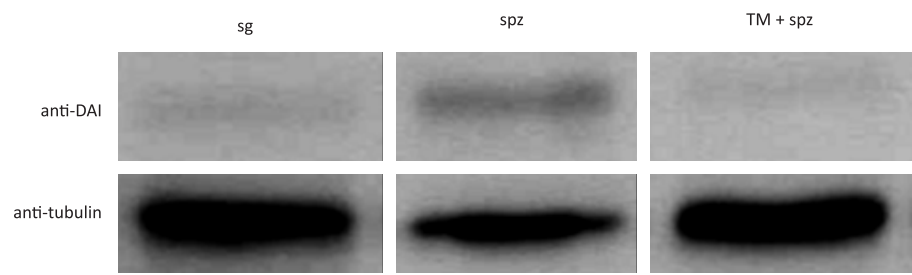


FIGURE 29

Suppression of ISG protein in TM treated mice.

Analysis by western blotting of ISG (DAI) protein expression induced by Spz infection in sg, spz or TM + spz infected mice. Tubulin expression was examined as protein loading control.

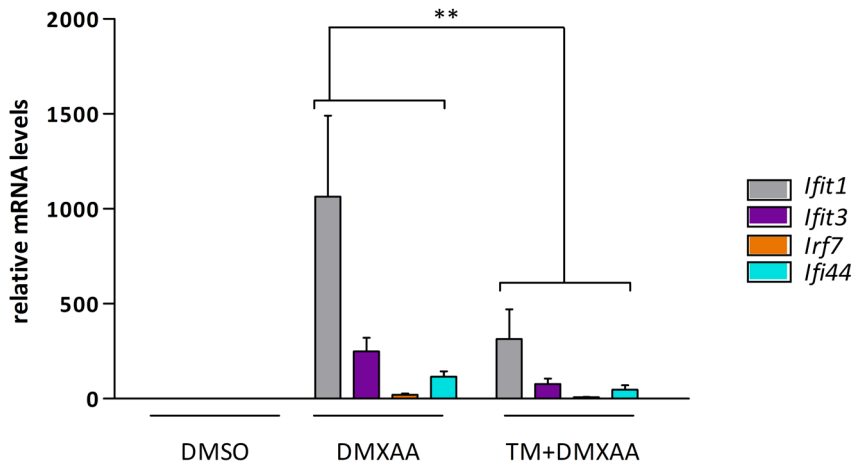


FIGURE 30

Suppression of interferon type-I response chemically induced with DMXAA in TM treated mice.

Real-time PCR analysis of Interferon Stimulated Genes (ISGs) (*Ifit1*, *Ifi44*, *Ifit3*, *Irf7*) mRNA in mice livers treated for 4h with DMXAA and mice livers treated for 8h TM plus 4h DMXAA. ** $p < 0.01$, t test. Results are expressed as means $\pm$ s.d.

genetically deficient mice for type I IFN receptor 1 (*Ifnr1* $-/-$) and pre-stimulated with TM or DMSO. Liver infection was quantified at two different time points, 24 hours and 44 hours pi. At 24 hours pi, while WT mice treated with TM show a significant increase in parasite liver load when compared to its DMSO control, this increase is not significant in *Ifnr1* $-/-$ (FIGURE 31). Later in infection, a more than 3 fold increase is observed in TM treated WT mice, while *Ifnr1* $-/-$ TM mice induces a 2 fold increase in liver infection (FIGURE 32). Together these findings demonstrate that the increase in liver infection in TM treated mice is partially dependent on an impaired type I IFN response and that this response is active earlier in infection. This is also suggested when we analyzed the fold increase at 24 and 44 hours pi between WT and *Ifnr1* $-/-$ (FIGURE 33). Here, while the liver infection burden significantly increases in *Ifnr1* $-/-$ at both time points when compared to its WT control, no significant difference is observed between *Ifnr1* $-/-$. This suggests that it is early in infection (until 24 hours) that the type I interferon response is actively killing the parasite.

In summary, our data shows that *Plasmodium berghei* liver infection induces ER stress and modulates UPR pathways; *in vivo* pharmacologically induced UPR activation leads to a significant increase in EEF number that can be observed at early stages of infection (before 24 hours); our results also show that ER stress induced increase in infection is partially dependent on interferon type I response.

Thus we concluded that UPR pathways activated by TM are enhancing *Plasmodium berghei* liver infection. However, due to the complexity of UPR signalling and because TM induces all UPR pathways, the study of individual pathways in the liver is crucial to understand how UPR and *Plasmodium* infection crosstalk to determine *in vivo* liver infection.

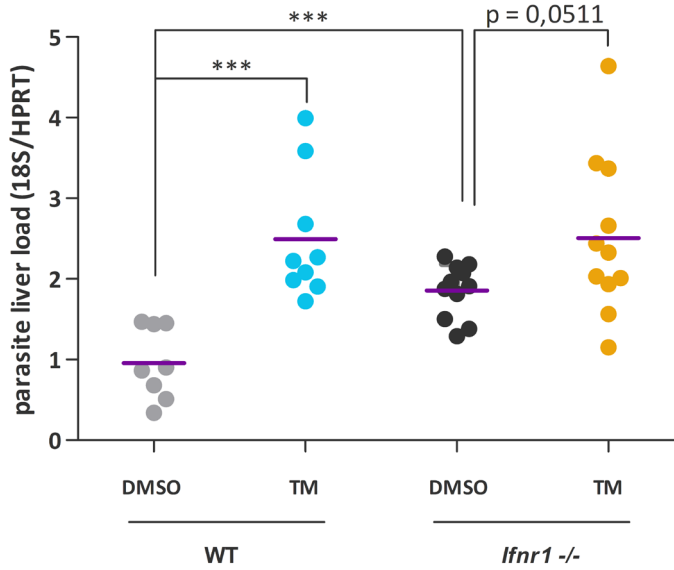


FIGURE 31

Infection increase in TM treated livers is dependent on interferon-type I response at earlier stages of liver infection.

Plasmodium berghei (Pb) liver load quantification at 24h after i.v. sporozoite injection on WT (C57BL/6) and *Ifnr1*^{-/-} mice treated with DMSO (control) or tunicamycin (TM) for 8 hours. Infection measured by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hppt) *** $p < 0,001$, t test. Results are expressed as means $\pm$ s.d. ($n \geq 4$ per group) ($n=2$ independent experiments).

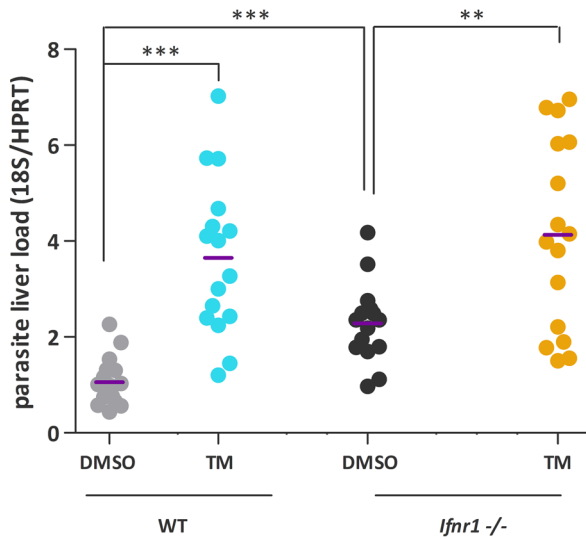


FIGURE 32

Infection increase in TM treated livers is partially dependent on interferon-type I response at later stages of liver infection.

Plasmodium berghei (Pb) liver load quantification at 44h after i.v. sporozoite injection on WT (C57BL/6) and *Ifnr1*^{-/-} mice treated with DMSO (control) or tunicamycin (TM) for 8 hours. Infection measured by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hpert) *** $p < 0.001$, t test. Results are expressed as means $\pm$ s.d. ($n \geq 5$ per group) ($n=3$ independent experiments).

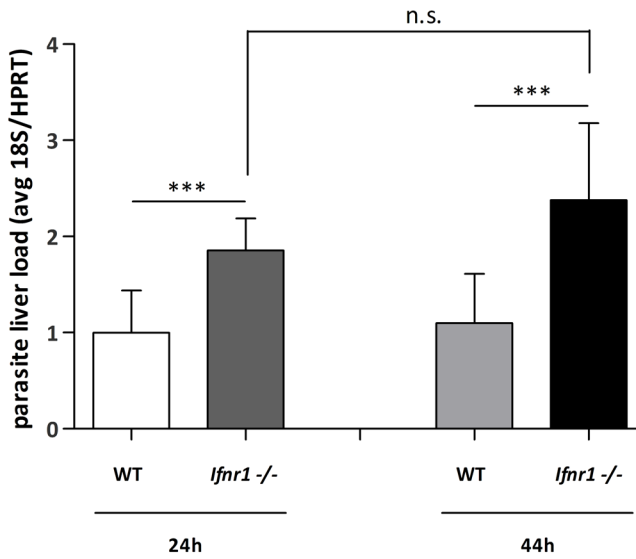


FIGURE 33

Type I IFN response kills *Plasmodium* early in infection.

Plasmodium berghei (Pb) liver load quantification at 24h and 44h after i.v. sporozoite injection on WT (C57BL/6) and *Ifnr1*^{-/-} mice. Infection measured by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hprt) *** p < 0.001, t test. Results are expressed as means ± s.d. (n ≥ 5 per group) (n = 3 independent experiments).

4.2 Impact of discrete UPR pathways on *Plasmodium* liver infection

4.2.1 The XBP1 pathway

XBP1 deletion in the liver significantly impairs *Plasmodium* infection XBP1 role in highly metabolic organs, particularly in the liver has been extensively reported ([192], [147], [193]). To test if *Plasmodium* infection induces XBP1 maturation we infected Hepa 1.6 cells with GFP-expressing *P. berghei* ANKA parasites and purified infected cells by FACS at 6 and 24 hours pi. Hepa 1.6 GFP-negative cells, from each time point, were used as control for basal mRNA expression. We found that *Plasmodium* infection induces the maturation of XBP1 to its active form, XBP1s, together with the increased expression of its target gene *Dnajb9* at both time-points analyzed (FIGURE 34). In Albuquerque *et al.*, *Dnajb9* is significantly induced at 6 hours pi while XBP1 expression is down-regulated after 18 hours pi (FIGURE 18), however in this study the maturation of XBP1 mRNA was not investigated. Moreover, the fold changes have been suggested to be under-estimated in the microarray results [61] which may account for the differences observed.

To understand the role of XBP1 in malaria liver infection, we infected mice bearing an inducible, conditional disruption of the XBP1 gene in the liver with *P. berghei* sporozoites and assessed the outcome of infection. We observed a significant decrease in liver infection in mice without XBP1 (f/f) when compared to its heterozygous control (f/+) (FIGURE 35A, B). This phenotype is a consequence of a reduction in the number of infected cells (FIGURE 36). Furthermore, in mice infected with XBP1s-expressing adenovirus (Ad-XBP1s) *Plasmodium* liver load was increased (FIGURE 37). This result is, however, preliminary since only one experiment was still performed and needs confirmation.

Altogether, our results suggest a direct effect of XBP1 regulated pathways in *Plasmodium* liver infection.

XBP1 deletion in the liver significantly down-regulates the expression of critical lipogenic genes

In the liver, XBP1 was shown to regulate hepatic lipogenesis independently of being an ER stress-response mediator [147]. As reported, we found that XBP1 deletion in the liver significantly down-regulated the expression of critical lipogenic genes as diacylglycerol acetyltransferase 2 (*Dgat2*), stearyl coenzyme A (CoA) desaturase 1 (*Scd1*) and acetyl CoA carboxylase 2 (*Acc2*) (FIGURE 38).

Therefore, our results suggest that an active engagement of XBP1 mediated *de novo* lipid synthesis might be an important pathway to the establishment of a successful liver infection by *Plasmodium*.

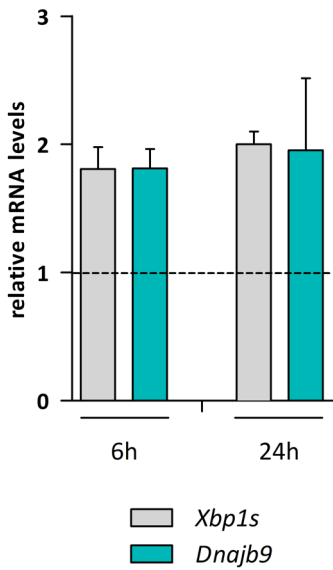
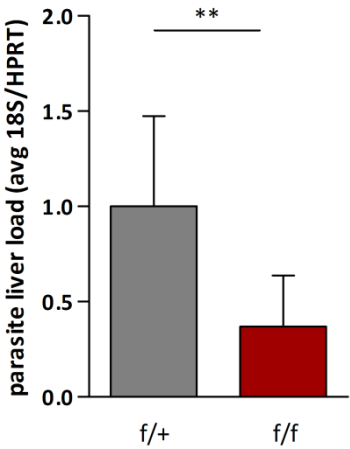


FIGURE 34
***Plasmodium* infection induces upregulation of XBP1 active form and XBP1s target gene *Dnajb9*.**
 Real-time PCR analysis of *Xbp1-S* and *Dnajb9* mRNA in sorted Hepa 1.6 cells infected with *P. berghei* at 6 and 24 hours post-infection. Normalization to its respective time-point salivary gland control (---). Normalization with two housekeeping genes (GAPDH/HPRT) Results are expressed as means $\pm$ s.m. (n=2 independent experiments).

A



B

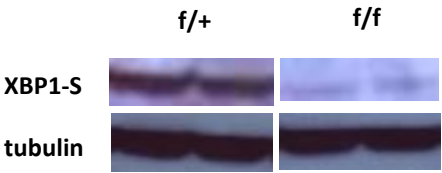


FIGURE 35

XBP1 deletion in the liver significantly impairs *Plasmodium* infection.

Plasmodium berghei (Pb) liver load quantification at 42h after i.v. sporozoite injection on mice bearing and inducible conditional disruption of the XBP1 gene in the liver (f/f) by qPCR. Normalization to the heterozygous litter mates control (f/+) *** $p < 0.001$, t test. Results are expressed as means $\pm$ s.d. ($n \geq 4$ per group) ($n=3$ independent experiments) (A) Western blot of nuclear XBP1s in XBP1 Δ mice (f/f) and control mice (f/+) (B).

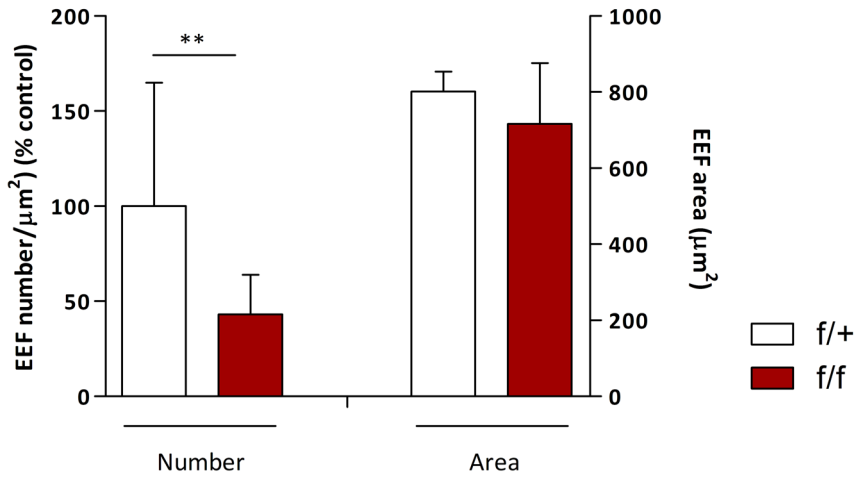


FIGURE 36

XBP1 deletion in the liver decreases parasite numbers.

Fluorescent microscopy quantification of EEf numbers and development (EEf area) on Xbp1 f/+ and Xbp1 f/f mice at 42h after sporozoite injection ** p < 0.01, t test. (n ≥ 6 per group) (liver slices ≥ 12 per mouse) Results are expressed as means ± s.d. (n = 3 independent experiments).

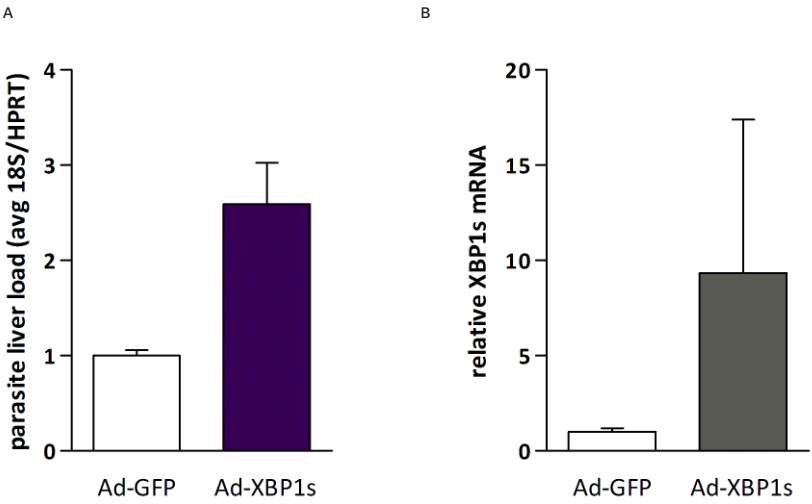


FIGURE 37
Infection increase in mice infected with XBP-1s-expressing adenovirus (Ad-XBP1s). *Plasmodium berghei* (Pb) liver load quantification (A) and relative expression of *XBP1s* mRNA (B) at 42h after i.v. sporozoite injection on mice infected with Ad-GFP or Ad-XBP1s 48h before sporozoite injection ($n \geq 3$ per group).

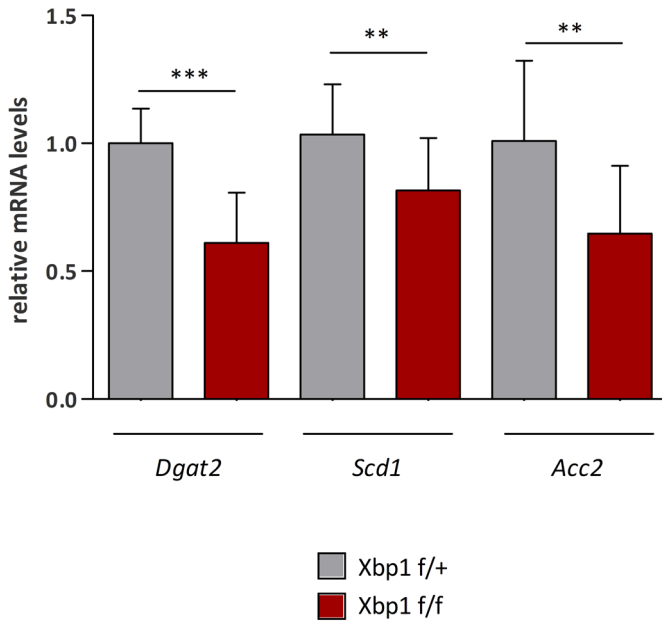


FIGURE 38

XBP1 deletion significantly down-regulates the expression of critical lipogenic genes.

Expression of lipogenic genes in the liver of *Xbp1 f/+* and *f/f* mice ** $p < 0,01$ *** $p < 0,001$, t test. Results are expressed as means $\pm$ s.d. ($n \geq 4$ per group) ($n = 3$ independent experiments).

4.2.2 The CREBH pathway

CREBH mRNA expression is induced in *Plasmodium* infection

CREBH is an endoplasmic reticulum-bound transcription factor specifically and highly expressed in the liver and small intestine [138] and is activated upon ER stress. We tested if during *Plasmodium* infection CREBH mRNA is induced. In Hepa 1.6 sorted cells at 6 and 24 hours pi, CREBH mRNA expression was highly upregulated at both time points analysed (FIGURE 39).

CREBH knockdown in primary hepatocytes significantly decreases *Plasmodium* infection and impairs hepcidin expression

To understand the functional significance of CREBH induced expression in liver infection, we infected mouse primary hepatocytes with adenovirus-expressing small hairpin RNA for CREBH (Ad-CREBHi) before sporozoite infection. A significant decrease in infection was observed upon CREBH knockdown (FIGURE 40). Previous reports have linked *Plasmodium* infection with iron availability both in hepatocytes ([194],[195]) and erythrocytes [196]. Interestingly, CREBH was shown to act as a regulator of iron homeostasis, through hepcidin induction, in response to ER stress [136]. More recently, hepatic gluconeogenesis was also reported as being regulated by CREBH expression in the liver [137]. To identify the downstream effectors of CREBH, we investigated the levels of expression of hepcidin (*Hamp*) and the gluconeogenic genes *G6Pase*, *PEPCK-C* and *PGC1 α* in Ad-CREBHi infected primary hepatocytes. While hepcidin expression was severely impaired in CREBH knockdown cells (FIGURE 41A), we observed no decrease in the expression of glucose related genes (FIGURE 41B).

Iron administration rescues CREBH mediated decrease in infection

Hepcidin controls cellular iron export by binding to ferroportin, an iron exporter present at the surface of hepatocytes among other cells, and triggering its internalization and degradation [197], therefore blocking iron efflux from hepatocytes. We sought to determine if the CREBH-Hamp mediated knockdown, and hence iron efflux from hepatocytes, was the cause of the reduction in infection in the absence of CREBH. For that, we supplemented primary hepatocytes depleted of CREBH with iron using ferric ammonium citrate (FAC). In the absence of FAC, primary hepatocytes depleted of CREBH exhibited a significant decrease in infection. However, primary hepatocytes depleted of CREBH but supplemented with iron had no changes in infection (FIGURE 42). These results clearly show that CREBH mediated hepcidin expression remodels iron availability in hepatocytes, which negatively impacts *Plasmodium* infection. Previous reports have shown that

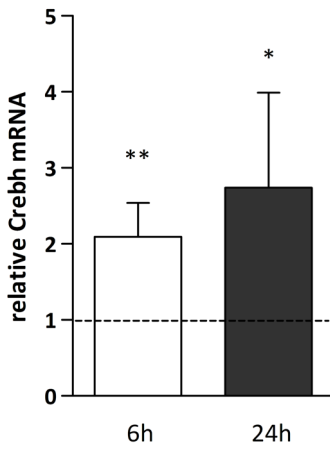


FIGURE 39

Induction of Crebh mRNA expression by *Plasmodium berghei* infection.

Real-time PCR analysis of hepatic *Crebh* mRNA in sorted Hepa 1.6 cells infected with *P. berghei* and analysed at 6 and 24 hours post-infection. Normalization to its respective time-point salivary gland control (---) * $p < 0,05$ ** $p < 0,01$, t test. Results are expressed as means $\pm$ s.m. (n=5 independent experiments).

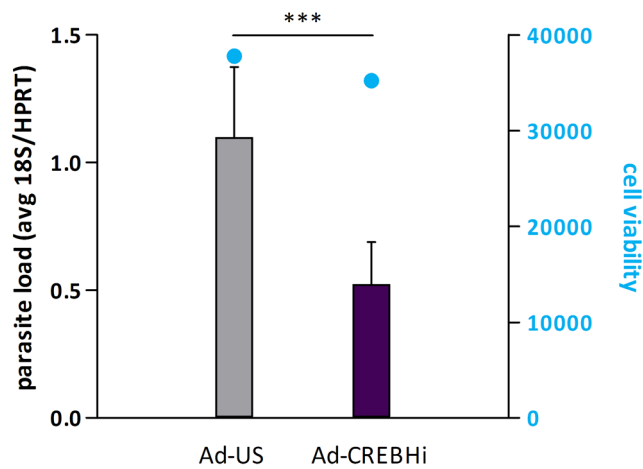


FIGURE 40
Crebh knockdown in primary hepatocytes significantly decreases *Plasmodium berghei* infection.

Mouse primary hepatocytes infected with adenovirus-expressing small hairpin RNA for CREBH (Ad CREBH RNAi) and a control adenovirus (Ad-US) were infected with *P. berghei* sporozoites and infection measured at 44 hours by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (Hprt) expression. Cell viability was measured by Alamar Blue assay (fluorescence intensity). Results are expressed as means $\pm$ s.d, *** $p < 0.001$, t test. (n=3 independent experiments).

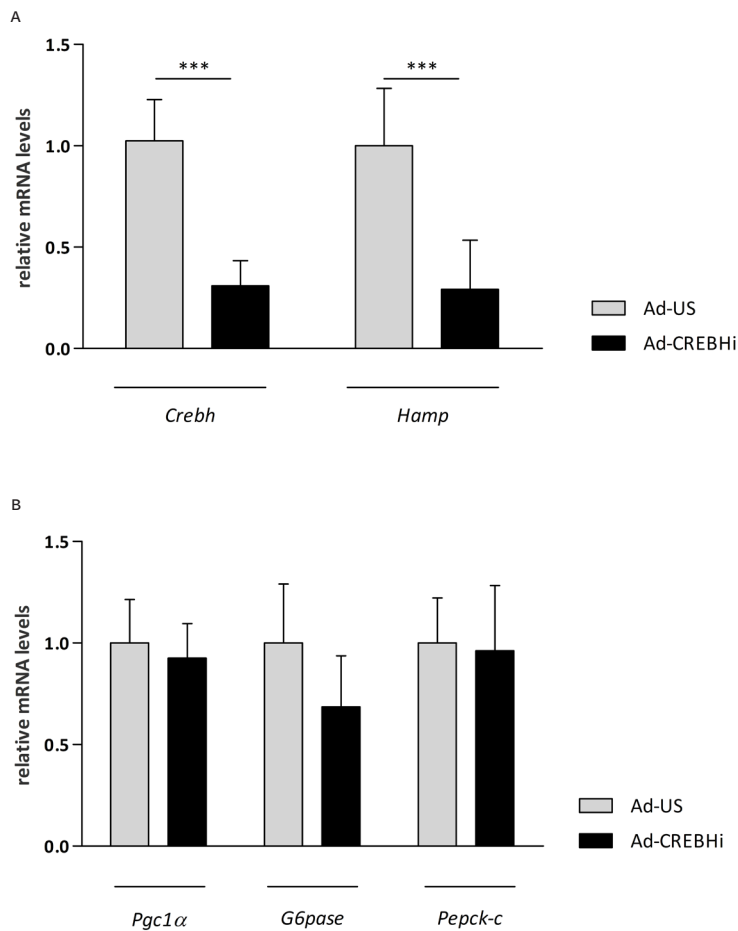


FIGURE 41
**Crebh knockdown significantly impairs
Hepcidin expression in primary
hepatocytes.**
Real time PCR analysis of mouse primary
hepatocytes infected with adenovirus-
expressing small hairpin RNA for CREBH (Ad
CREBH RNAi) and a control adenovirus (Ad-
US) for *Crebh* and *Hamp* mRNA expression
(A) and for gluconeogenesis genes *PGC1α*,
G6Pase and *PEPCK-C* (B).

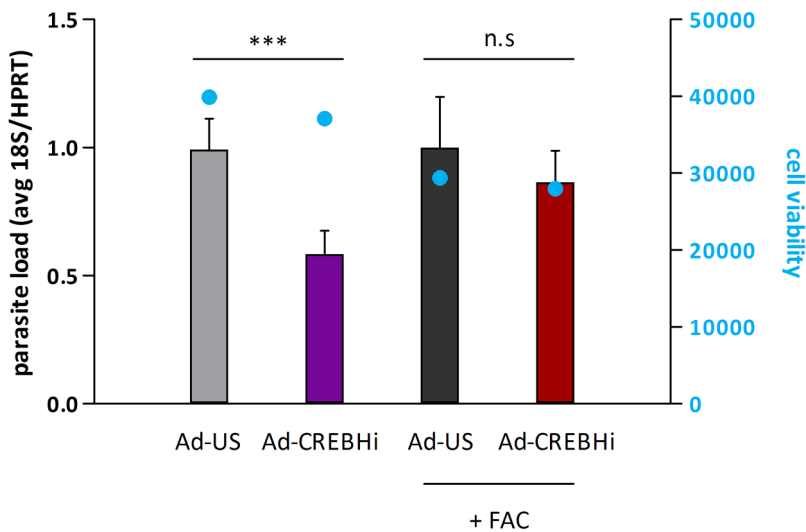


FIGURE 42

Rescue of infection phenotype in CREBH knockdown to control levels with FAC supplemented medium in primary hepatocytes.

Mouse primary hepatocytes infected with adenovirus-expressing small hairpin RNA for CREBH (Ad-CREBH RNAi) and a control adenovirus (Ad-US) were infected with *P. berghei* sporozoites and infection measured at 44 hours by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (Hprt) expression. Cell viability was measured by Alamar Blue assay (fluorescence intensity). Results are expressed as means $\pm$ s.d., *** $p < 0.001$, t test ($n=3$ independent experiments).

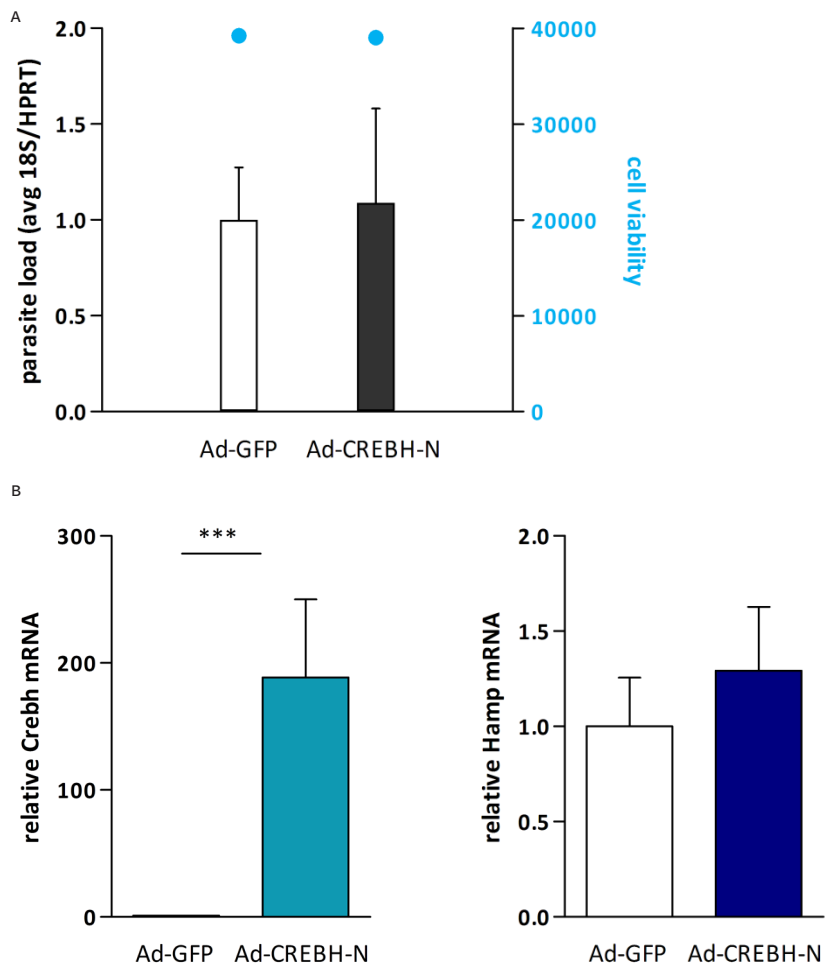


FIGURE 43
CREBH overexpression in primary hepatocytes has no effect on *P. berghei* infection.

Mouse primary hepatocytes infected with adenovirus for nuclear CREBH (Ad CREBH-N) were infected with *P. berghei* sporozoites. Infection was measured at 44 hours by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (Hprt) expression (A) Relative expression of *Crebh* and *Hamp* mRNA (B) Cell viability was measured by Alamar Blue assay (fluorescence intensity). Results are expressed as means $\pm$ s.d., *** $p < 0.001$, t test ($n=3$ independent experiments).

iron supplementation increases infection [194]. We tested if by overexpressing CREBH we would have a higher parasite burden. We infected primary hepatocytes with the active form of CREBH (Ad CREBH-N) previous to sporozoite infection. Although we were able to overexpress CREBH no significant increase was observed in hepcidin expression. Hence, we also did not see any increase in infection (FIGURE 43A, B). This suggests that a plateau in hepcidin expression is probably reached or that cells have mechanisms to counterbalance this demand.

***In vivo* CREBH depletion reduces *Plasmodium* infection**

To determine the role of CREBH in an *in vivo* model of infection, we infected mice with the same adenovirus (Ad-CREBHi for knockdown and the Ad-CREBH-N for overexpression). Similarly to our results *ex vivo*, CREBH *in vivo* depletion significantly impairs hepcidin expression and a reduction in parasite liver load is observed (FIGURE 44A, B). CREBH overexpression, while successful in induction of hepcidin transcription, had no effect in liver infection (FIGURE 45).

Our findings suggest that the specific liver UPR branch mediated by CREBH acts as a novel and key mediator of iron availability to *Plasmodium* during liver infection, by regulating the hormone hepcidin.

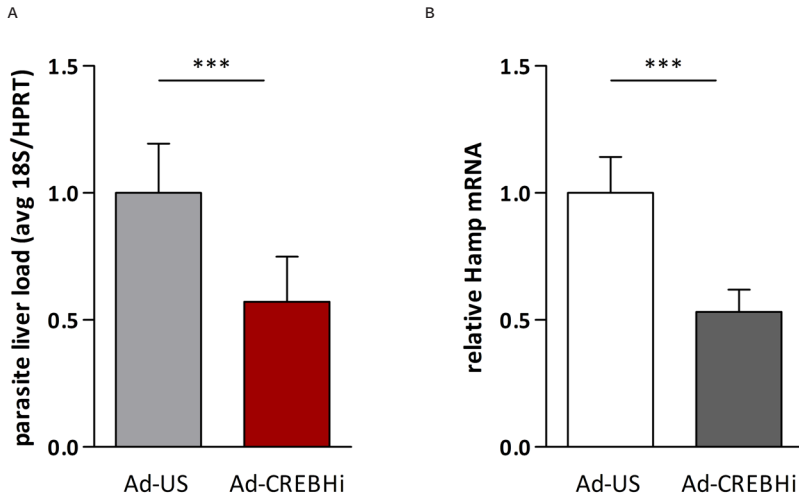


FIGURE 44

***In vivo* CREBH knockdown decreases *Plasmodium berghei* liver infection.**

Plasmodium berghei (Pb) liver load quantification at 44 hours after i.v. sporozoite injection in C57BL/6 infected with Ad-US and Ad-CREBHi for 48 hours prior to spz infection. Infection measured by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hpert) (A) *Hamp* mRNA expression measured by qPCR in mice infected with Ad-US and Ad-CREBHi for 48h prior to spz infection (B) *** p < 0.001, t test. Results are expressed as means $\pm$ s.d. (n $\geq$ 4 per group) (n=2 independent experiments).

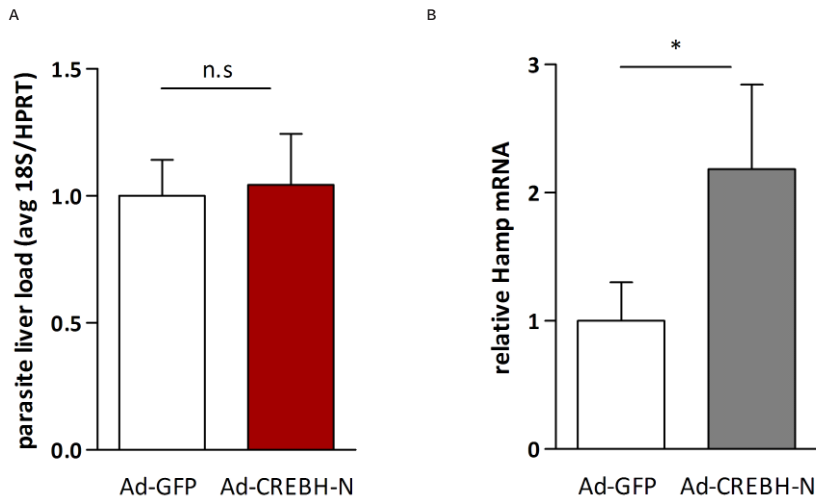


FIGURE 45

***In vivo* CREBH overexpression has no effect on *P. berghei* liver infection.**

Plasmodium berghei (Pb) liver load quantification at 44h after i.v. sporozoite injection in C57BL/6 infected with Ad-US and Ad-CREBH-N for 48h prior to spz infection. Infection measured by real-time PCR for parasite 18S ribosomal RNA normalized with hypoxanthine-guanine phosphoribosyltransferase (hpert) (A) *Hamp* mRNA expression measured by qPCR in mice infected with Ad-GFP and Ad-CREBH-N for 48h prior to spz infection (B) * $p < 0,05$ ** $p < 0,01$, t test. Results are expressed as means $\pm$ s.d. (n=5 per group).

DISCUSSION

CHAPTER THREE

During their development within hepatocytes, *Plasmodium* parasites establish a replicative niche that culminates in the production of thousands of new parasites. This massive replication creates a challenging environment within hepatocytes and ultimately to their endoplasmic reticulum (ER) functions. However, how *Plasmodium* intrahepatic development impacts on ER homeostasis has never been addressed. In this thesis, we demonstrate for the first time that *Plasmodium* infection induces ER stress in hepatocytes concomitantly with UPR activation pathways, which impacts on the outcome of the infection. We showed, at both transcriptional and protein level, that *Plasmodium* infection induces the expression of specific ER stress markers, as the proteins BiP and PDI, together with the unfolded protein response (UPR) mediators *Chop*, *Atf4* and *Herpud1* and ultimately confirmed by an hepatic ER landscape marked by an increase in the protein folding burden.

Such an ER stress response, which reflects the loss of ER homeostasis, has been associated with several different infectious diseases, mainly those caused by enveloped viruses ([198],[199],[200]) but also with certain bacteria ([165],[201, 202]), through the impact of the activation of UPR in multiple pathways. One of the pathways modulated by UPR is innate immunity; however, UPR activation impacts differently on innate immune responses to intracellular pathogens. In *C. elegans*, the IRE1 α -XBP1 and, to a lesser extent, the ATF6 branches of the UPR are important for defense against bacterial pore-forming toxins (PFTs), since loss of XBP1 and ATF6 rendered animals 3 to 6 times more susceptible to PFTs, respectively [165]. On the other hand, TLR4 and TLR2 were shown to activate the IRE1 α -XBP1 branch in macrophages, causing a sustained production of the cytokines IL-6 (interleukin 6) and TNF- α (tumor necrosis factor-alpha) [163]. *In vitro* and *in vivo* innate immune-dependent XBP1 response against the TLR2-activating human intracellular pathogen *Francisella tularensis* further confirmed the TLR-XBP1 activation in mammalian host defences [203]. However, while UPR branches can intersect and enhance innate immune responses to orchestrate protection against the aforementioned bacteria, studies with viral

infections revealed the opposite, with disruption of antiviral innate immune response being mediated by UPR signalling branches. From the vast array of viruses that activate the UPR, one of the most notable ones is the hepatitis C virus (HCV), which targets the liver as primary organ of infection, as *Plasmodium*, and can result in chronic infections, liver cirrhosis and ultimately hepatocellular carcinoma [204]. Recently, HCV was found to induce the UPR, which in turn activated the autophagic pathway [205]. Several studies have shown that autophagy is activated in conditions of ER stress and that the UPR can specifically be induced to activate the formation of the autophagosome ([206],[108]). The authors found that UPR-mediated autophagy activation promoted HCV replication by inhibiting innate immunity. Specifically, the classical three UPR sensors together with CHOP and other UPR-autophagy molecules, as ATF5 and LC3B, were shown to suppress the HCV PAMP-triggered activation of IFN- β [205]. HCV had previously been shown to block interferon effector function through activation of the integrated stress response (ISR) [189]. In the liver, HCV induces a strong type I interferon-stimulated gene (ISG) expression. However, despite this response, the HCV infection persists. The authors found that above a certain threshold, HCV triggered the phosphorylation of eIF2 α , through PKR, which in turn attenuates ISG protein expression despite the ISG mRNA induction [189]. Type I IFNs act on an autocrine and paracrine manner to engage the IFN type I cell surface receptor (IFNAR), which is composed of two subunits – IFNAR1 and IFNAR2 [207]. HCV and vesicular stomatitis virus (VSV) were shown to promote the IFNAR1 ubiquitination and degradation through UPR activation, namely the PERK branch [190]. This mechanism represents another way of suppressing type I IFN response and to perpetrate viral efficient replication. Thus, UPR signalling pathways have been shown to restrict but also promote innate immune responses against intracellular pathogens.

In order to understand how ER stress activation impacts *Plasmodium* liver infection, we induced *in vivo* ER stress prior to sporozoite infection with a sublethal intraperitoneal injection tunicamycin (TM). TM induces ER stress through inhibition of asparagine (N)-linked glycosylation, which causes the accumulation of malfolded proteins in the ER lumen [208]. We found that activation of ER stress in the liver significantly increased the parasite hepatic burden. The more than 3 fold increase is solely attributed to a higher number of exoerythrocytic forms (EEF) in the liver, which is evident early in infection (14-24 hours).

Given the role of UPR activation in blocking innate immune responses against other intracellular pathogens ([205], [189], [190]), our results suggested that UPR could enhance *Plasmodium* liver infection by interfering with an innate immune response against the parasite. However, while the role of innate immunity in infections with intracellular pathogens as vi-

ruses, bacteria and a few protozoan parasites ([209], [210]) is well established, its role during malaria infection has only recently been studied and the focus of research directed to the blood-stage of infection (reviewed in [211]). Indeed, there is a paucity of information regarding the role of innate immunity in malaria liver infection [212], with questions such as if *Plasmodium* is detected and eliminated by an innate immune response, and if the parasite is able to subvert this response, remaining unanswered. Here, by investigating the mechanisms by which ER stress activation enhances *Plasmodium* liver infection, we found that *Plasmodium berghei* induces a type I interferon response. There is a clear induction of IFN- β mRNA expression and interferon-stimulated genes (ISGs). The observed induction of the type I IFN response associates with a higher susceptibility to *Plasmodium* in *Ifnr1* $-/-$ mice, already early in infection (until 24 hours pi). In fact, our results suggest that *Plasmodium berghei* is sensitive to the type I IFN response specifically within this time window, since we observed no significant difference in parasite liver load in *Ifnr1* $-/-$ mice at later stages (between 24 - 44 hours pi). Our results are thus in agreement with earlier work which suggested that *Plasmodium berghei*, as well as *Plasmodium yoelli* (albeit with differences in intensity and timing) induce an inflammatory response in the liver ([213], [214], [185]).

The type I interferon response was decreased during *in vivo* ER stress, as a significant impairment in ISGs induction is observed upon TM treatment. This decrease could even be achieved in the absence of infection, as 8 hours of TM treatment alone already impacted ISGs basal expression and chemically induction of ISGs by DMXAA, a potent inducer of type I IFN [191] was also significantly decreased. Therefore, ER stress activation per se can negatively impact a type I IFN response. However, the mechanism responsible for this negative regulation is not yet understood, as different factors might account to it:

- (a) both mouse [215] and human ([216],[217]) type I interferons carry N-glycosylation sites, and TM treatment has been proposed to block interferon activity in mice [218], due to decreased IFN glycosylation and thereby decreasing its activity [215];
- (b) ER stress inducers, such as brefeldin A (BFA) and TM can block IFN mediated STAT1 phosphorylation ([219],[220]) and STAT1 trafficking to the nucleus [220], which is a vital step in type I IFN signalling;
- (c) *in vitro* studies showed that UPR inducers, as thapsigargin (TG) and TM but also viral infections induced PERK-mediated phosphorylation of IFNAR1 within its degron (a motif that mediates proteolytic turnover), leading to IFNAR1 ubiquitination and degradation and, concomitantly, suppression of type I IFN response ([190], [221]).

Regardless of the specific mechanism, in *Ifnr1* $-/-$ mice ER stress activation resulted in a tendency to increase (although not significant) in infection at 24 hours, while later in infection parasite liver load is still significantly high. Collectively, these results demonstrate that while ER stress activation impacts *Plasmodium* liver infection by suppressing innate immunity its effects are not limited to this response, as late in infection livers lacking a type I IFN response still exhibit higher parasite load (see ahead the discussion on this matter).

Despite *Plasmodium berghei* inducing and being susceptible to a type I IFN response in the liver infection is still established, suggesting that there are parasites that are able to suppress or subvert this response. Indeed, several parasite immune evasion strategies have been reported ([60]). One of these mechanisms is the induction, by the parasite, of the host anti-inflammatory enzyme HO-1, which decreases the number of recruited inflammatory cells late in infection [185]. Interestingly, HO-1 is an endoplasmic reticulum-anchored enzyme [222] that is induced upon ER stress [223] however, no impaired recruitment was found with UPR activation. In fact, parasite's elimination within 24 hours of infection does not depend on inflammatory cell recruitment, since no quantitative difference in inflammatory cells in the liver was observed between the different groups – no infection, infected and UPR activation plus infection. Thus, *Plasmodium berghei* is eliminated by liver-resident cells other than hepatocytes, at least during the first 24 hours of infection. So far, despite our efforts, due to its extremely low number and heterogeneous activation patterns, we were not able to identify which liver cells become activated and are responsible for the parasite elimination.

Following the results of this study, we propose that UPR activation by *Plasmodium berghei* acts as a new immune evasion strategy due to its negative regulation on innate immune responses, but further studies are required to understand the exact mechanism(s) involved. The ability to evade the host immune system correlates with the replication rate of the parasite. Accordingly, within *Plasmodium* rodent species, *Plasmodium yoelii* induces less inflammation [224] and produces more EEFs than *Plasmodium berghei* [64] and has been suggested to represent a suitable model for human malaria [225]. Therefore, it is reasonable to posit that this particular aspect of UPR and innate immunity should be investigated in other *Plasmodium* species.

In the liver, *Plasmodium* parasites undergo a massive replication where a bulk of lipids are required to support the generation of thousands of merozoites. While *Plasmodium* possesses a type II fatty acid biosynthetic pathway [226], several studies showed that in the liver the parasite relies on its own pathway only at the transition stage from the liver to the blood ([227],[228],[229]). Therefore, during liver stage parasite lipid needs must

be fulfilled by the host. However, which host-mediated lipid molecules as well as which pathways are required during malaria liver stage remain largely elusive.

In line with our previous results, and to understand how individual UPR pathways enhance *Plasmodium* infection, we showed that XBP1 mRNA was spliced in cells infected with *Plasmodium berghei*, together with induction of the known XBP1s target gene *Dnajb9*. *In vivo* experiments in mice bearing a specific deletion of XBP1 in the liver showed that XBP1 was important for infection. Notably, significantly fewer parasites were found in XBP1 knockout livers, while preliminary data suggest that adenovirus-mediated XBP1s overexpression resulted in higher liver parasite load. The ER has a central role in the regulation of lipid metabolism and all UPR pathways have been implicated in the regulation of lipogenesis [230]. In the liver, XBP1s was shown to regulate the expression of genes involved in fatty acid synthesis [147]. As reported, we found a significant impairment in the transcription of the same genes, including *Scd-1* (stearoyl-CoA desaturase-1), *Acc2* (acetyl-CoA carboxylases 2) and *Dgat2* (diacylglycerol acetyltransferase 2) in XBP1 knockout livers, thus establishing a functional link between XBP1-mediated regulation of lipid lipogenesis and infection. Two recent studies also demonstrated that XBP1s induces the synthesis of phospholipids, mainly phosphatidylcholine (PC), the primary phospholipid of eukaryotic membranes in general, and the ER membrane in particular [99]; also it contributed to the proliferation of cellular membranes, leading to ER expansion as well as increased cell size [98]. SCD1 is a key regulatory enzyme in the biosynthesis of oleate and palmitoleate, which are the major monounsaturated fatty acids of membrane phospholipids, triglycerides (TAG), and cholesterol esters (CE) [231]; DGAT2 catalyzes the final reaction in the synthesis of TAG and in the liver, SCD1 and DGAT2 colocalize in the ER membrane where SCD1 channels the monounsaturated fatty acids to TAG synthesis [232]; ACC2 catalyzes the synthesis of malonyl-CoA, which is the crucial substrate for the biosynthesis of long chain fatty acids [233]. Thus, our results suggest that one or several products in *de novo* hepatic fatty acid synthesis are required during *Plasmodium* liver infection and that XBP1 activation in the liver might be a way to obtain it. Understanding the contribution of each step will allow us a more detailed map of the interaction of liver *de novo* lipogenesis with the parasite. We hypothesize, however, that impaired phospholipid synthesis in XBP1 knockout livers might account for the decrease in infection, most importantly the PC content, since it constitutes the major phospholipid in membranes. This is particularly important, since each of the thousands of merozoites are surrounded by an individual membrane and recent studies showed that the membrane surrounding merozoites is host cell derived [234].

Plasmodium tropism for hepatocytes suggests that liver specific factors may be exploited by the parasite to its own benefit. Interestingly, CREBH is highly and selectively expressed only in the liver and the small intestine ([135], [138]). CREBH levels increase in response to proinflammatory cytokines and inducers of ER stress ([135],[235]). Here we showed that *Plasmodium berghei* induces CREBH expression since early in infection. In agreement with previous reports [136] we found that CREBH regulates the expression of hepcidin (Hamp), the major regulator of mammalian iron homeostasis. Hepcidin, a circulating regulatory hormone peptide produced primarily by hepatocytes, controls cellular iron export by regulating the amount of ferroportin, an iron exporter present on the surface of duodenal enterocytes, hepatocytes, macrophages and placental cells [197]. Our findings have demonstrated that adenovirus-mediated CREBH knockdown in mouse primary hepatocytes cultures reduced *Plasmodium* infection along with a markedly decrease in Hamp expression. The observed phenotype suggested that iron efflux, mediated by reduced hepcidin expression and therefore iron release by ferroportin from hepatocytes impaired infection. Iron is an essential nutrient that has been shown to be required during *Plasmodium* liver infection ([194], [195]). In fact, microarray analysis showed that *Plasmodium* infected hepatoma cells significantly increased the iron importer divalent metal transporter-1 (DMT1), while ferroportin expression was highly reduced [61]. Here we showed that iron supplementation to primary hepatocytes reversed the effects of adenovirus-mediated CREBH-Hamp decrease. Thus, our results strongly suggest the parasite's dependence on CREBH-Hamp pathway to fulfill its iron requirements. Mammalian iron homeostasis is balanced by two regulatory systems, one that functions at a cellular level and other systemically, the latest relying on hepcidin and ferroportin. *In vivo* CREBH down-modulation impaired hepcidin expression which resulted in reduced parasite liver load, therefore supporting our hypothesis that loss of CREBH expression, both *ex vivo* and *in vivo*, by adenovirus mediated knock-down reduces infection by decreasing liver hepcidin expression and, as a consequence, leading to iron efflux from hepatocytes. A previous study suggested that increased iron availability both *in vitro* as *in vivo* enhances infection [194]. Given our results, we examined the role of CREBH-Hamp axis overexpression in infection. Surprisingly, we did not observe Hamp induction with adenovirus-mediated expression of CREBH active fragment (CREBH-N) in mouse primary hepatocytes, as previously reported [136]. However, our preliminary *in vivo* data showed that a significant increase in Hamp expression, by overexpression of CREBH-N, did not increase liver infection. Since expression of ferroportin is already decreased in infected cells [61], our results suggest that the parasite is capable of efficiently manipulate this pathway for iron acquisition. Hepcidin increase was

found to be associated with several infectious diseases [236]. While this represents an iron-withholding mechanism from circulating pathogens it enhances the risk for intracellular pathogens, as *Plasmodium*. This particular branch of UPR in the liver, mediated by CREBH, thus represents another mechanism whose manipulation clearly enhances *Plasmodium* liver infection.

ER stress and UPR activation were first described in infectious diseases with viruses. In virus infected cells, the host translational machinery is used by the infecting virus to produce large amounts of viral proteins, which disrupts ER homeostasis leading to ER stress [237]. Our work has shown that during liver stage infection *Plasmodium* induces ER stress with UPR activation. A relevant question is, therefore, what is the nature of disrupted ER homeostasis mechanisms in the context of malaria liver infection? We hypothesize several possible mechanisms. Emerging evidence indicates that viruses and bacteria can manipulate the UPR directly by secreting virulence factors ([237],[202],[238],[239]). With the notable exception of *Plasmodium* infection of host erythrocytes [240], still little is known about how the parasite manipulates the host cell during liver stage infection and whether parasite factors directly interfere with host pathways, namely UPR. Approximately 75% of proteins are synthesized in the ER and all secreted and membrane proteins that enter the secretory pathway are checked for proper folding in the ER lumen [241]. This represents an extraordinary task, especially in a highly metabolic active organ as the liver, where hepatocytes have an extremely high protein synthesis rate of approximately 13 million secretory proteins, per minute [242]. Thus, in hepatocytes even subtle perturbations to protein synthesis can lead to ER stress. Additionally, alterations in ER fatty acid/lipid composition might induce ER stress ([243], [144], [230]). Our study together with Albuquerque *et al.* showed that *Plasmodium* liver infection impacts profoundly the metabolic status of hepatocytes, as with fatty acid metabolism, and so hepatic lipid alterations induced by malaria liver infection might also account to ER stress.

CONCLUSIONS AND FUTURE CHALLENGES

CHAPTER FOUR

The liver stage represents the first and obligatory phase of malaria infection where the parasite, within a membrane bound vacuole, replicates culminating in the generation of thousands of new infectious parasites. The work developed in this Doctoral Thesis shed light on a crucial and unknown aspect of the cell biology of *Plasmodium*-liver interaction that clearly regulates the infection outcome. While *Plasmodium* is an obligate intracellular pathogen, its development in the liver impacts hepatic endoplasmic reticulum (ER) homeostasis, leading to ER stress and inducing the activation of the unfolded protein response (UPR). We also discovered that *Plasmodium*, while in the liver, induces and is highly susceptible to a type I interferon response, which UPR activation clearly abrogates, therefore establishing UPR as a potential new mechanism of immune evasion. Our study also revealed that the UPR regulated pathways by XBP1 and CREBH remodels the metabolic hepatic repertoire, lipid and iron metabolism respectively, with a significant impact to malaria liver infection. The fact that our work was performed *in vivo* represents a new glimpse to the intricate complexity of *Plasmodium*-liver interaction with clearly clinical relevance.

Plasmodium is an infectious agent, however, our work showed that in the liver *Plasmodium* infection impacts on a host pathway, UPR, common to metabolic diseases, e.g obesity and type II diabetes, suggesting that the liver stage could also be viewed, in certain aspects, as a metabolic disease. Therefore, our results emphasize the importance of considering malaria liver infection in the context of host metabolism for future clinical approaches. Interestingly, the ER is being considered an attractive potential therapeutic target, under the premise that maintaining ER function and reducing ER stress may be able to prevent metabolic diseases. Therefore, in light of our results, we postulate that resolving ER stress in the liver can mitigate malaria liver infection.

The work here developed, while enlightened for a novel aspect of *Plasmodium*-liver biology, important aspects of ER-*Plasmodium* interaction are unknown. The UPR is a complex pathway mediated, in the liver, by four dif-

ferent ER sensors. Therefore, the role of PERK-eIF2 α and ATF6 pathways in malaria liver infection is still to be determined. We showed that *Plasmodium* activates a type I interferon response, however, how the parasite triggers this response is unknown. Recently, the IRE1 branch of the UPR was shown to sense bacterial proteins invading the ER and to activate an innate immune response. If *Plasmodium* directly targets the ER is thus an interesting question to be addressed. Moreover, our work suggests that UPR activation can abrogate a type I interferon response. To determine which UPR pathway(s) are responsible for the mitigation of this innate immune response is, therefore, instrumental to fight liver stage malaria.

MATERIALS AND METHODS

CHAPTER FIVE

Chemicals. RPMI 1640, DMEM, William's E, PBS pH 7.4, Trypsin, were purchased from Gibco/Invitrogen. Tunicamycin (TM), DMSO and FAC (ferric ammonium citrate) were purchased from Sigma. Percoll from GE Healthcare. Alamar Blue from Invitrogen.

Antibodies. Anti-calnexin (Santa-Cruz), rabbit antiserum against UIS4 kindly provided by Stephan H. I. Kappe; Hoechst and AlexaFluor-conjugated secondary antibodies from Molecular Probes/Invitrogen; anti-XBP1 (ABCAM); anti-DAI (kindly provided by Andreas Pichlmair).

Cells. Huh7 cells, a human hepatoma cell line, were cultured in RPMI 1640 medium supplemented with 10% heat-activated Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin solution, 1% HEPES, 1% Non-essential amino acids, and 1% Glutamine in a 5% CO₂ humidified incubator at 37°C. Hepa 1.6 cells, a mouse hepatoma cell line, and the 293A cell line (Invitrogen) were cultured in DMEM medium supplemented with 10% heat-activated Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin solution, 1% HEPES, 1% Non-essential amino acids, and 1% Glutamine in a 5% CO₂ humidified incubator at 37°C. All supplements were purchased from Gibco/Invitrogen.

Isolation of murine primary hepatocytes and culture conditions. Mouse primary hepatocytes were isolated as previously described [244]. Briefly, hepatocytes were obtained by perfusion of mouse liver lobules with liver perfusion medium (Gibco/Invitrogen) and liver digest medium (Gibco/Invitrogen) at 37°C using a peristaltic pump. Hepatocytes were then purified using a 1.12 g/ml, 1.08 g/ml and 1.06g/ml Percoll gradient. Cells were cultured in William's E medium containing 4% FBS, 1% pen/strep in 24-well plates coated with 0.2% Gelatine in PBS. Cells were maintained in culture at 37°C and 5% CO₂. Hepatocytes were obtained from male C57BL/6 mice (6-8weeks). Hepatocytes were seeded at 100 000 cells per well (24-well

plates) and cell confluency was determined by Alamar Blue fluorescence method. Briefly, cells were incubated with 1/20 dilution of Alamar Blue in complete William's E medium for about 1-2 hours and the fluorescence was measured at 595 nm in a microplate reader (Tecan). Following the determination of cell confluency, cells were rinsed briefly with PBS and supplemented with William's E complete medium.

Parasite strains and mice. GFP-expressing *Plasmodium berghei* (parasite line 259cl2) [169] and luciferase-expressing *Plasmodium berghei* (parasite line 676m1cl1 line) [182] sporozoites were obtained from the dissection of infected female *Anopheles stephensi* mosquito salivary glands between days 20 and 24 days after the infectious blood meal. Sporozoite number was determined by counting in a Neubauer chamber. C57BL/6 mice (male) were obtained from Charles River and all experiments performed with mice from 6-8 weeks. *Ifnr1* ^{-/-} mice and C57BL/6 *Ifnr1* background control mice were obtained from the Instituto Gulbenkian de Ciência animal house facility. In each experiment, each mouse group was formed with mice from the same age range, gender and supplier, to exclude variation within groups.

Real time *in vivo* luminescence measurement of *Plasmodium berghei* liver infection. Mice infected with luciferase-expressing *Plasmodium berghei* sporozoites at 14, 24 and 42 hours post-infection were imaged as previously described [182]. Briefly, mice were anesthetized with isoflurane (IsoFlo, Veterinária Esteves) and immediately injected subcutaneously (in the neck) with D-Luciferin dissolved in PBS (0,15 mg/g, Caliper Life Sciences). Precisely 5 min after, mice were intra-peritoneally (i.p.) injected with 200 µl of an anaesthesia mixture comprising Ketamine (1,2% of Imalgene 1000, Merial) and Xylazine (0,8% of 2% Rompun, Bayer) diluted in PBS, and placed inside the imaging camera of the *in vivo* Imaging System (IVIS® Lumina Imaging System). Bioluminescence imaging was acquired at 10 minutes after D-Luciferin injection, with a 12.5 cm field of view (FOV), binning factor of 4 and an exposure time of 180 seconds. Quantitative analysis of bioluminescence was performed by measuring the luminescence signal intensity using the region of interest (ROI) settings of the Living Image® Software (version 3.0). The ROIs were set to measure the abdominal area (that was previously shaved using chemical depilatory, Veet®) at the location of the liver. ROI measurements were expressed in total flux of photons, and were used to infer liver stage infection.

Mosquito bite infection. Mice were intra-peritoneally injected with 200 µl of anaesthesia mixture (80 mg/kg Ketamine and 10 mg/kg Xylazine) diluted in PBS. Each mouse was exposed to *Anopheles stephensi* mosquitoes infected with GFP-expressing *Plasmodium berghei* for 30 minutes.

Quantification of parasite liver load by quantitative real-time PCR (qRT-PCR). Livers were collected at the mentioned time-point of infection and homogenized in denaturing solution (4 M guanidine thiocyanate; 25mM sodium citrate pH 7, 0.5% N-Lauroylsarcosine and, 0.7% β -Mercaptoethanol in DEPC-treated water). Total RNA was extracted using RNeasy Mini kit (Qiagen). 1 μ g of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche) with the following cycle: 25°C for 10 minutes, 55°C for 30 minutes, and 85°C for 5 minutes. Infection load in the liver was determined by qRT-PCR using PbA 18S rRNA primers and normalization against the Hypoxanthine Guanine Phosphoribosyltransferase (*Hprt*) housekeeping gene. qRT-PCR was performed in a 7500 Fast Applied Bioscience (AB) machine and ViiA™ 7 Real-Time PCR System (both Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute; melting stage was done at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 30 seconds. Primers sequences in Table 1.

Fluorescence activated cell sorting (FACS) of *Plasmodium berghei* infected and uninfected cells. Human hepatoma cells, Huh7, were seeded in 12 wells plates and incubated overnight in a 37°C incubator with 5% CO₂ level. The cells were subsequently infected with GFP-expressing *Plasmodium berghei* sporozoites. At different time points of infection (2 and 12 hours), the cells were trypsinized for 5 minutes at 37°C and resuspended in an appropriate volume of 10% FBS supplemented PBS. The cells were pelleted by centrifugation at 1200 rpm for 5 minutes and further resuspended in an appropriate volume of 2% FBS-supplemented PBS. Non-infected and GFP-expressing *Plasmodium berghei* infected cells were gated on the basis of their different fluorescence intensity, as previously established [61]. The cells were FACs sorted in BD High Speed Cell Sorter ARIA I using the 100 μ m nozzle. Before sorting the cells were passed by a 100 μ m nozzle. The GFP positive (GFP +) cells were collected sequentially and the same amount of non infected (GFP negative) cells were collected from each sample, therefore a control for each GFP + population was obtained. Immediately after sorting, the cells were pelleted and snap-frozen in liquid nitrogen and stored at -80°C until the samples were analysed. This procedure was performed repeatedly until collected roughly one million sorted-infected cells per time-point.

Immunohistochemistry of mouse liver slices and EEFs quantification.

Livers infected with GFP-expressing *Plasmodium berghei* sporozoites were harvested at 42 hours post-infection, fixed in 4% PFA for 30 minutes at room-temperature, washed with PBS three times (5 minutes each time) and then sliced into 50 μm sections using a vibratome (VT1000S, Leica). Sections were permeabilized and blocked overnight (ON) at 4°C in 0,3% Triton X-100 and 1% Bovine Serum Albumin (Sigma). After permeabilization/blocking, sections were incubated ON at 4°C in blocking solution with anti-GFP IgG Alexa fluor 488 conjugate AB (Invitrogen), alexa 594 phalloidin (Invitrogen) and 4',6-diamino-2-phenylindole (DAPI, Sigma). Final wash with PBS three times (5 minutes each time) and sections were then mounted on a slide, digitalized (CanoScan lide 60, Canon) and the areas measured using Image J. EEFs were counted on Zeiss Axiovert 200M inverted widefield fluorescence microscope.

Immunofluorescence. Hepa 1.6 infected cells were fixed in 4% paraformaldehyde (wt/vol) for 10 min at room temperature, permeabilized/blocking in 0.1% Triton X-100-2% BSA (wt/vol), and incubated with 1° antibodies (anti-Calnexin/anti-UIS4) for 1h at room-temperature. After washing with PBS, appropriate 2° antibodies were added and incubated for 1h. Nuclei were stained with Hoechst, for 5 minutes. Coverslips were mounted in Fluoromount. All images were acquired on Zeiss confocal microscopes.

Fluorescence activated cell sorting (FACS) of *Plasmodium berghei* infected and non-infected cells for gene expression analysis. Mouse hepatoma cells, Hepa 1.6, were seeded in 12 wells plates and incubated overnight in a 37°C incubator with 5% CO₂ level. The cells were subsequently infected with GFP-expressing *Plasmodium berghei* sporozoites. At different time points of infection (6 and 24 hours), the cells were trypsinized for 5 minutes at 37°C and resuspended in an appropriate volume of 10% FBS supplemented PBS. The cells were pelleted by centrifugation at 1200 rpm for 5 minutes and further resuspended in an appropriate volume of 10% FBS-supplemented PBS. Non-infected and GFP-expressing *Plasmodium berghei* infected cells were gated on the basis of their different fluorescence intensity, as previously established [61]. The cells were FACs sorted in BD High Speed Cell Sorter ARIA I or ARIA III using the 100 μm nozzle. Before sorting the cells were passed by a 100 μm nozzle. The GFP positive (GFP +) cells were collected sequentially and the same amount of non infected (GFP negative) cells were collected from each sample, therefore a control for each GFP + population was obtained. Immediately after sorting, the cells were pelleted and snap-frozen in liquid nitrogen and stored at -80°C until the samples were analysed.

Gene expression quantification by qRT-PCR of sorted *Plasmodium berghei* infected and uninfected cells. In snap-frozen sorted-infected and non infected cells total RNA was extracted with the RNeasy Mini Kit (Qia-gen). Total RNA was converted to cDNA with the SuperScript III Reverse Transcriptase (Invitrogen) with the following cycle: 65°C for 5 minutes then placed on ice for at least 1 minute; 50°C for 50 minutes, and 85°C for 5 minutes. Chill on ice. Collect the reactions by brief centrifugation and treat with RNase H for 20 min at 37°C. Gene expression of mouse *Atf4*, *Chop*, *Atf3*, *Herpud1*, *Xbp1s*, *Dnajb9* and *Crebh* was determined by qRT-PCR using specific primers and normalization against the *Hprt* and/or *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) housekeeping genes. qRT-PCR was performed in ViiA™ 7 Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute; melting stage was done at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 30 seconds. Primers sequences in Table 1.

PCR for splicing XBP1 assay. Total RNAs were used for the first-strand synthesis with Transcriptor First Strand cDNA Synthesis kit (Roche) with the following cycle: 25°C for 10 minutes, 55°C for 30 minutes, and 85°C for 5 minutes. PCR primers (5'-ACACGCTTGGGAATGGACAC-3' and 5'-CCATGGGAAGATGTTCTGGG-3') encompassing the missing sequences in *Xbp1s* was used for the PCR amplification with PCR Master Mix (Promega). PCR products were separated by electrophoresis on a 3% agarose gel and visualized by gel red staining.

Western blot. Briefly, a liver lobe was homogenized in 1 ml of homogenization buffer (50 mM Tris-HCL, pH 7.6, 2% SDS supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche) and PhosSTOP (Phosphatase Inhibitor Cocktail Tablets, Roche) with a Polytron homogenizer and immediately aliquoted and stored at -80°C. The liver homogenate (100 – 200 µl) was then lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, supplemented with Protease and Phosphatase Cocktail Inhibitors immediately before lysis). Lysates were briefly sonicated and cleared by centrifugation for 5 min. Protein quantification was performed using the Bradford method. Western blot analyses on liver extracts were performed with rabbit anti-XBP1 (ABCAM) that recognizes the spliced form of XBP1 (spliced form confirmed in our blot by positive control cells with TM treatment) and anti-DAI (kindly provided by Andreas Pichlmair).

Adenovirus. Adenovirus for CREBH RNAi (Ad-CREBHi) and its control (Ad-US) and for nuclear CREBH (Ad-CREBH-N) and its control (Ad-GFP) were kindly provided by Seung-Hoi Koo (Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Korea). Adenovirus for XBP1s (Ad-XBP1s) with its control virus (Ad-GFP) were kindly provided by Ann-Hwee Lee (Weill Cornell Medical College, New York). Amplification and purification of high-titer recombinant adenoviruses was performed as previously described [245]. Final purification and concentration was performed with Vivaspin 20 MW 100 KDa (Sartorius) according to manufacturer's instructions. Viral titer was determined by infecting HEK-293A cells with serially diluted viral stock and counting GFP positive cells 24 hours after infection by FACS.

Adenovirus *in vivo* and *ex vivo* infection. Mice were infected with 1×10^8 GFPu /g of body weight for each adenovirus (Ad-US/Ad-CREBHi, Ad-GFP/Ad-CREBH-N, Ad-GFP/Ad-XBP1s) by tail-vein injection 48h prior to sporozoite infection. All mice were then infected with 5×10^4 *Plasmodium berghei* sporozoites and 42-44 hours later livers were collected and liver infection was determined by expression of PbA 18S rRNA. Primary hepatocytes were infected with the recombinant adenoviruses at 10 GFPu per cell 24 hours prior to sporozoite infection. Infection was determined at 44 hours by expression of PbA 18S rRNA. GFPu (GFP positive units).

Mice bearing an inducible, conditional disruption of the Xbp1 gene in the liver genotyping. Mice harbouring Mx1-CRE/loxP sites were kindly provided by David Fidock (Department of Microbiology&Immunology, Columbia University, New York). Mice were generated as previously described [147]. All experiments with XBP1 mice were performed at Columbia University under the guidelines of the Columbia University Animal Facility. Mouse genotyping was performed using DNA from mouse tail extracted with DNease Blood & Tissue Kit (Qiagen). PCR genotyping was performed with primers for the floxed allele (flox), Cre and IL2 (PCR control) primers (Table 1) with the following cycle: 94°C for 3 minutes, 94°C for 30 seconds/56°C for 30 seconds/72°C for 30 seconds (35 cycles), 72°C 5 minutes.

Tunicamycin injection. Tunicamycin diluted in 150 mM dextrose at 50 µg/ml was intraperitoneally injected at 1 µg /g body weight.

Analysis of proteomics screen. The raw data with the intensities for all identified proteins was used to the analysis. In the raw data file, a filter system was applied to the possible missing values obtained during the mass

spectrometry measurement. We kept only proteins that have no missing values in at least 2 samples. The missing values were then replaced with the minimum value for each sample. The data was then normalized with the TMM method [1]. We performed the analysis between 12 hours data set (GFP positive versus GFP negative) with R package “limma”[2]. In short, this statistical method tried to capture the mean-variance relation in the data to determine whether a gene is differentially expressed (DE) in two groups of samples. We considered as statistical significant the DE proteins analysed by the previous method with corrected p-value ≤ 0.05 .

[1] Oshlack, Alicia, and Mark D Robinson. “A Scaling Normalization Method for Differential Expression Analysis of RNA-seq Data.” 11 (3): R25.

[2] Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* 3, No. 1, Article 3.

Statistical analysis. Statistical analysis were performed using GraphPad Prism 5 software. Student t-test was used for significance with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

TABLE 1
Real-time PCR primers used for mRNA quantification

Target	Forward sequence	Reverse sequence
P. berghei ANKA r18S	AAGCATTAATAAAGCGAATACATCCTTAC	GGAGATTGGTTTGTGACGTTTATGTG
human/mouse HPRT	TTTGCTGACCTGCTGGATTAC	CAAGACATTCTTCCAGTTAAAGTTG
GAPDH	AACCTTGGCATTGTGGAAGC	ACACATTGGGGGTAGGAACA
mouse ATF4	CGGCCTAAGCCATGGCGCTC	AGCCCCCAACCCGACTGTG
mouse CHOP	GGTGGCAGCGACAGAGCCAG	GCACGTGGACCAGGTTCTGCTT
mouse ATF3	CCAGGTCTCTGCCTCAGAAG	TCCAGGGGCTGTGTTGTAC
mouse Herpud1	TGGACCTATTCCGCAGCGACG	CCACCCGACGTGGTGCAGGT
mouse Mgl2	GGATCCCAAAATTCCCAATT	TCCCTCTTCTCCAGTGTGCT
mouse Klrb1	GTTTCCAACACTTGGGAGGA	CCAGTTCATGTCTGGCAATG
mouse Ncf2	GCAGTGGCCTACTTCCAGAG	ATTCTAGTTGGTTGCCAATG
mouse CD3	TCTGGGAAGTCGAGGACAGT	ATCAGCAAGCCAGAGTGAT
mouse CD19	GAGAAAGCTGGCTTGGTATCG	CTGACGTCTGAAGCAATCCA
mouse Ifit1	CCTTTACAGCAACCATGGGAGA	GCAGCTTCCATGTGAAGTGAC
mouse Rlg-I	CCACCTACATCCTCAGCTACATGA	TGGGCCCTTGTGTTCTCTT
mouse Stat1	GACCCTAAGCGAATCGGATAC	TGTCGCCAGAGAGAAATTCGTGT
mouse Usp18	CGTGCTTGAGAGGGTCAATTG	GGTCGGGAGTCCACAACCTTC
mouse Ifit2	AGAGGAAGAGGTTGCCTGGA	CTCGTTGTACTCATGACTGCTG
mouse Ifit3	CTGAACCTGCTCAGCCACAC	TGGACATACCTCTCCCTGA
mouse Ifi44	TGGAATTCATGAACCAATCAC	CAAATGCAGAATGCCATGTTTT
mouse Lgp2	TCATCTGTACGGCAGAGTTGT	TGTTGTAGACGGTGCTTTTGT
mouse Mx2	AGACAAAGCATGGCACTTCC	ACTGGATGATCAAGGGAACG
mouse Irf7	CTTCAGCACTTTCTCCGAGA	TGTAGTGTGGTGACCTTGC
mouse IFN- β	TCCAAGAAAGGACGAACATTGG	TGAGGACATCTCCACGTCAA
mouse Xbp1s	GAGTCCGCAGCAGGTG	GTGTGAGATCCATGGGA
mouse Dnajb9	CGTCGGGGCGCAGAGTTATT	TCGCTCTGAGCAGAGACTTTGGC
mouse Dgat2	TTCTGCGCATAAGGCCCTATT	AGTCTATGGTGTCTCGGTTGAC
mouse Scd1	TCTGCGCTCTTACCCCTTG	AAACCTGCCCTCTGACTCT
mouse Acc2	GGGCTCCCTGGATGACAAC	TTCCGGGAGGAGTTCTGGA
mouse Crebh	AGCGGGGACTTGCAACAGCA	GAGAGGCAGCTGGGTGGGGA
mouse Hamp	TGAGCAGACCACTATCTC	ACTGGGAATTGTTACAGCAATT
mouse Pgc1 α	AACACAACGAACAAGGGCGAC	TCAGCAGTGTCTTTAGGGTGG
mouse G6pase	ACGCCCTATTGGTGGGTCT	GCCAGAGGACTTCTGTGTCG
mouse Pepck-c	GCCGAGACTAGCGATGGGGGT	ATGGTTCCGCTCTCGTGGT

REFERENCES

FIGURES REFERENCES

Albuquerque, S. S., C. Carret, A. R. Grosso, A. S. Tarun, X. Peng, S. H. Kappe, M. Prudencio and M. M. Mota (2009). "Host cell transcriptional profiling during malaria liver stage infection reveals a coordinated and sequential set of biological events." BMC Genomics 10: 270.

Courselaud, B., C. Pigeon, Y. Inoue, J. Inoue, F. J. Gonzalez, P. Leroyer, D. Gilot, K. Boudjema, C. Guguen-Guillouzo, P. Brissot, O. Loreal and G. Ilyin (2002). "C/EBPalpha regulates hepatic transcription of hepcidin, an anti-microbial peptide and regulator of iron metabolism. Cross-talk between C/EBP pathway and iron metabolism." J Biol Chem 277(43): 41163-41170.

Fu, S., S. M. Watkins and G. S. Hotamisligil (2012). "The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling." Cell Metab 15(5): 623-634.

Goldberg DE, C. A. (2010). "Moving in and renovating: exporting proteins from Plasmodium into host erythrocytes." Nat Rev Microbiol. 8(9): 617-621.

Hall JC, Rosen A. (2010). "Type I interferons: crucial participants in disease amplification in autoimmunity." Nat Rev Rheumatol. 6(1):40-9.

Hentze, M. W., M. U. Muckenthaler, B. Galy and C. Camaschella (2010). "Two to tango: regulation of Mammalian iron metabolism." Cell 142(1): 24-38.

Hetz C, M. F., Rodriguez D, Glimcher LH. (2011). "The Unfolded Protein Response: Integrating Stress Signals Through the Stress Sensor IRE1α." Physiol Rev. 91(4)(1219): 1219-1243.

Hotamisligil, G. S. and E. Erbay (2008). "Nutrient sensing and inflammation in metabolic diseases." Nat Rev Immunol 8(12): 923-934.

Kaufman, R. J., D. Scheuner, M. Schroder, X. Shen, K. Lee, C. Y. Liu and S. M. Arnold (2002). "The unfolded protein response in nutrient sensing and differentiation." Nat Rev Mol Cell Biol 3(6): 411-421.

Nicolas, G., C. Chauvet, L. Viatte, J. L. Danan, X. Bigard, I. Devaux, C. Beaumont, A. Kahn and S. Vaulont (2002). "The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation." Journal of Clinical Investigation 110(7): 1037-1044.

Oliveira SJ, P. J., Picarote G, Costa VM, Carvalho F, Rangel M, de Sousa M, de Almeida SF. (2009). "ER stress-inducible factor CHOP affects the expression of hepcidin by modulating C/EBPalpha activity." PLoS One. 4(8): e6618.

Pigeon, C., G. Ilyin, B. Courselaud, P. Leroyer, B. Turlin, P. Brissot and O. Loreal (2001). "A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload." J Biol Chem 276(11): 7811-7819.

Prudêncio M, R. A., Mota MM. (2006). "The silent path to thousands of merozoites: the Plasmodium liver stage." Nat Rev Microbiol. 4(11): 849-856.

Riley, E. M. and V. A. Stewart (2013). "Immune mechanisms in malaria: new insights in vaccine development." Nat Med 19(2): 168-178.

Ron, D. and J. F. Habener (1992). "CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription." Genes & Development 6(3): 439-453.

Ron, D. and P. Walter (2007). "Signal integration in the endoplasmic reticulum unfolded protein response." Nat Rev Mol Cell Biol 8(7): 519-529.

Vecchi, C., G. Montosi, K. Zhang, I. Lamberti, S. A. Duncan, R. J. Kaufman and A. Pietrangelo (2009). "ER stress controls iron metabolism through induction of hepcidin." Science 325(5942): 877-880.

TEXT REFERENCES

- 1 World Health Organization. *World malaria report 2012*. World Health Organization. http://www.who.int/malaria/publications/world_malaria_report_2012/wmr2012_full_report.pdf 2012.
- 2 Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ et al., *Global malaria mortality between 1980 and 2010: a systematic analysis*. Lancet, 2012. **379**(9814): p. 413-431.
- 3 RTS,S Clinical Trials Partnership, Agnandji ST, Lell B, Fernandes JF, Abosolo BP et al., *A Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Infants*. N Engl J Med., 2012. **367**(24): p. 2284-95.
- 4 Olotu A, Fegan G., Wambua J, Nyangweso G, Awuondo KO, et al., *Four-Year Efficacy of RTS,S/AS01E and Its Interaction with Malaria Exposure*. N Engl J Med., 2013. **368**(12): p. 1111-20.
- 5 Cogswell FB, *The hypnozoite and relapse in primate malaria*. Clin Microbiol Rev., 1992. **5**(1): p. 26-35.
- 6 Matuschewski K., *Getting infectious: formation and maturation of Plasmodium sporozoites in the Anopheles vector*. Cell Microbiol, 2006. **8**(10): p. 1547-56.
- 7 Cowman, AF and Crabb BS, *Invasion of red blood cells by malaria parasites*. Cell, 2006. **124**(4): p. 755-66.
- 8 Besteiro S, Dubremetz JF, Lebrun M, *The moving junction of apicomplexan parasites: a key structure for invasion*. Cell Microbiol. , 2011. **13**(6): p. 797-805.
- 9 Keeley A, Soldati D, *The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa*. Trends Cell Biol., 2004. **14**(10): p. 528-32.
- 10 Farrow RE, Green J, Katsimitsoulia Z, Taylor WR, Holder AA et al., *The mechanism of erythrocyte invasion by the malarial parasite, Plasmodium falciparum*. Semin Cell Dev Biol, 2011. **22**(9): p. 953-60.
- 11 Stubbs J, Simpson KM, Triglia T, Plouffe D, Tonkin CJ et al., *Molecular mechanism for switching of P. falciparum invasion pathways into human erythrocytes*. Science, 2005. **309**(5739): p. 1384-7.

- 12 Crosnier C, Bustamante LY, Bartholdson SJ, Bei AK, Theron M et al., *Basigin is a receptor essential for erythrocyte invasion by Plasmodium falciparum*. Nature, 2011. **480(7378)**: p. 534-7.
- 13 Douglas AD, Williams AR, Illingworth JJ, Kamuyu G, Biswas S et al., *The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody*. Nat Commun. , 2011. **2**: p. 601.
- 14 Glushakova S, Yin D, Li T, Zimmerberg J, *Membrane transformation during malaria parasite release from human red blood cells*. Curr Biol, 2005. **15(18)**: p. 1645-50.
- 15 Wickham ME, Culvenor JG and Cowman AF, *Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte*. J Biol Chem, 2003. **278(39)**: p. 37658-63.
- 16 Salmon, BL, Oksman A, and Goldberg DE, *Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis*. Proc Natl Acad Sci U S A, 2001. **98(1)**: p. 271-6.
- 17 Amino R, Thiberge S, Martin B, Celli S, Shorte S et al., *Quantitative imaging of Plasmodium transmission from mosquito to mammal*. Nat Med, 2006. **12(2)**: p. 220-4.
- 18 Sidjanski S, Vanderberg JP, *Delayed migration of Plasmodium sporozoites from the mosquito bite site to the blood*. Am J Trop Med Hyg. , 1997. **57(4)**(426-9): p. 426-9.
- 19 Vanderberg JP, Frevert U, *Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes*. Int J Parasitol, 2004. **34(9)**: p. 991-6.
- 20 Medica DL, Sinnis P, *Quantitative dynamics of Plasmodium yoelii sporozoite transmission by infected anopheline mosquitoes*. Infect Immun, 2005. **73(7)**: p. 4363-9.
- 21 Jin Y, Kebaier C, Vanderberg J, *Direct microscopic quantification of dynamics of Plasmodium berghei sporozoite transmission from mosquitoes to mice*. Infect Immun., 2007. **5(11)**: p. 5532-9.

- 22 Sinnis P, Zavala F, *The skin stage of malaria infection: biology and relevance to the malaria vaccine effort*. Future Microbiol. , 2008. **3(3)**: p. 275-8.
- 23 Shin SC, Vanderberg JP, Terzakis JA, *Direct infection of hepatocytes by sporozoites of Plasmodium berghei*. J Protozool., 1982. **29(3)**: p. 448-54.
- 24 Frevert U, Sinnis P, Cerami C, Shreffler W, Takacs B et al., *Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes*. J Exp Med., 1993. **177(5)**: p. 1287-98.
- 25 Sinnis P, Willnow TE, Briones MR, Herz J, Nussenzweig V, *Remnant lipoproteins inhibit malaria sporozoite invasion of hepatocytes*. J Exp Med., 1996. **84(3)**: p. 945-54.
- 26 Blouin A, Bolender RP, Weibel ER, *Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study*. J Cell Biol., 1977. **72(2)**: p. 441-55.
- 27 Pradel G, Garapaty S, Frevert U, *Proteoglycans mediate malaria sporozoite targeting to the liver*. Mol Microbiol., 2002. **45(3)**: p. 637-51.
- 28 Pradel G, Garapaty S, Frevert U, *Kupffer and stellate cell proteoglycans mediate malaria sporozoite targeting to the liver*. Comp Hepatol. , 2004. **3 (Suppl 1)**: p. S47.
- 29 Cerami C, Frevert U, Sinnis P, Takacs B, Clavijo P et al., *The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of plasmodium falciparum sporozoites*. Cell. , 1992. **70(6)**: p. 1021-33.
- 30 Lyon M, Deakin JA, Gallagher JT, *Liver heparan sulfate structure. A novel molecular design*. J Biol Chem., 1994. **269(15)**: p. 11208-15.
- 31 Pinzon-Ortiz C, Friedman J, Esko J, Sinnis P, *The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for plasmodium sporozoite attachment to target cells*. J Biol Chem., 2001. **276(29)**: p. 26784-91.
- 32 Coppi A, Tewari R, Bishop JR, Bennett BL, Lawrence RP et al., *Heparan sulfate proteoglycans provide a signal to Plasmodium sporozoites to stop migrating and productively invade host cells*. Cell Host Microbe, 2007. **2(5)**: p. 316-27.

- 33 Pradel G, Frevert U, *Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion*. Hepatology, 2001. **33**(5): p. 1154-65.
- 34 Ishino T, Chinzei Y, Yuda M, *A Plasmodium sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection*. Cell Microbiol., 2005 **7**(2): p. 199-208.
- 35 Ishino T, Yano K, Chinzei Y, Yuda M, *Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer*. 2004. **2**(1): p. E4.
- 36 Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U et al., *Migration of Plasmodium sporozoites through cells before infection*. Science, 2001. **291**(5501): p. 141-4.
- 37 Mota MM, Hafalla JC, Rodriguez A, *Migration through host cells activates Plasmodium sporozoites for infection*. Nat Med., 2002. **8**(11): p. 1318-22.
- 38 Carrolo M, Giordano S, Cabrita-Santos L, Corso S, Vigário AM et al., *Hepatocyte growth factor and its receptor are required for malaria infection*. Nat Med. , 2003. **9**(11): p. 1363-9.
- 39 Kariu T, Ishino T, Yano K, Chinzei Y, Yuda M, *CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts*. Mol Microbiol., 2006. **59**(5): p. 1369-79.
- 40 Silvie O, Franetich JF, Charrin S, Mueller MS, Siau A et al., *A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites*. J Biol Chem., 2004. **279**(10): p. 9490-6.
- 41 Ishino T, Chinzei Y, Yuda M, *Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte*. Mol Microbiol., 2005b. **58**(5): p. 1264-75.
- 42 van Dijk MR, Douradinha B, Franke-Fayard B, Heussler V, van Dooren MW et al., *Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells*. Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12194-9.
- 43 van Schaijk BC, Janse CJ, van Gemert GJ, van Dijk MR, Gego A et al., *Gene disruption of Plasmodium falciparum p52 results in attenuation of malaria liver stage development in cultured primary human hepatocytes*. PLoS One, 2008. **3**(10): p. e3549.

- 44 Jethwaney D, Lepore T, Hassan S, Mello K, Rangarajan R et al., *Fetuin-A, a hepatocyte-specific protein that binds Plasmodium berghei thrombospondin-related adhesive protein: a potential role in infectivity*. Infect Immun., 2005. **73(9)**: p. 5883-91.
- 45 Silvie O, Rubinstein E, Franetich JF, Prenant M, Belnoue E et al., *Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity*. Nat Med, 2003. **9(1)**: p. 93-6.
- 46 Silvie O, Charrin S, Billard M, Franetich JF, Clark KL et al., *Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites*. J Cell Sci., 2006. **119(Pt 10)**: p. 1992-2002.
- 47 Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F et al., *Binding of hepatitis C virus to CD81*. Science, 1998. **282(5390)**: p. 938-41.
- 48 Yalaoui S, Huby T, Franetich JF, Gego A, Rametti A et al., *Scavenger receptor BI boosts hepatocyte permissiveness to Plasmodium infection*. Cell Host Microbe, 2008. **4(3)**: p. 283-92.
- 49 Rodrigues CD, Hannus M, Prudêncio M, Martin C, Gonçalves LA et al., *Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection*. Cell Host Microbe, 2008. **4(3)**: p. 271-82.
- 50 Prudêncio M, Rodrigues CD, Hannus M, Martin C, Real E et al., *Kinome-wide RNAi screen implicates at least 5 host hepatocyte kinases in Plasmodium sporozoite infection*. PLoS Pathog, 2008. **4(11)**: p. e1000201.
- 51 Matuschewski K, Ross J, Brown SM, Kaiser K, Nussenzweig V et al., *Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage*. J Biol Chem, 2002. **277(44)**: p. 41948-53.
- 52 Mueller AK, Camargo N, Kaiser K, Andorfer C, Frevert U et al., *Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface*. Proc Natl Acad Sci U S A, 2005a. **102(8)**: p. 3022-7.
- 53 Mueller AK, Labaied M, Kappe SH, Matuschewski K, *Genetically modified Plasmodium parasites as a protective experimental malaria vaccine*. Nature, 2005b. **433(7022)**: p. 164-7.

- 54 Mikolajczak SA, Jacobs-Lorena V, MacKellar DC, Camargo N, Kappe SH, *L-FABP is a critical host factor for successful malaria liver stage development*. Int J Parasitol, 2007. **37**(5): p. 483-9.
- 55 Sharma A, Yogavel M, Akhouri RR, Gill J, Sharma A, *Crystal structure of soluble domain of malaria sporozoite protein UIS3 in complex with lipid*. J Biol Chem, 2008. **283**(35): p. 24077-88.
- 56 Favretto F, Assfalg M, Molinari H, D'Onofrio M, *Evidence from NMR interaction studies challenges the hypothesis of direct lipid transfer from L-FABP to malaria sporozoite protein UIS3*. Protein Sci, 2013. **22**(2): p. 133-8.
- 57 Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S et al., *Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids*. Science, 2006. **313**(5791): p. 1287-90.
- 58 Charoenvit Y, Mellouk S, Sedegah M, Toyoshima T, Leef MF et al., *Plasmodium yoelii: 17-kDa hepatic and erythrocytic stage protein is the target of an inhibitory monoclonal antibody*. Exp Parasitol. , 1995. **80**(3): p. 419-29.
- 59 Doolan DL, Hedstrom RC, Rogers WO, Charoenvit Y, Rogers M et al., *Identification and characterization of the protective hepatocyte erythrocyte protein 17 kDa gene of Plasmodium yoelii, homolog of Plasmodium falciparum exported protein 1*. J Biol Chem., 1996. **271**(30): p. 17861-8.
- 60 Singh AP, Buscaglia CA, Wang Q, Levay A, Nussenzweig DR et al., *Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite*. Cell, 2007. **131**(3): p. 492-504.
- 61 Albuquerque SS, Carret C, Grosso AR, Tarun AS, Peng X et al., *Host cell transcriptional profiling during malaria liver stage infection reveals a coordinated and sequential set of biological events*. BMC Genomics, 2009. **10**: p. 270.
- 62 Portugal S, Carret C, Recker M, Armitage AE, Gonçalves LA et al., *Host-mediated regulation of superinfection in malaria*. Nat Med, 2011. **17**(6): p. 732-7.
- 63 Sturm A, Graewe S, Franke-Fayard B, Retzlaff S, Bolte S et al., *Alteration of the parasite plasma membrane and the parasitophorous vacuole membrane during exo-erythrocytic development of malaria parasites*. Protist, 2009. **160**(1): p. 51-63.

- 64 Baer K, Klotz C, Kappe SH, Schnieder T, Frevert U, *Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature*. PLoS Pathog, 2007. **3**(11): p. e171.
- 65 Shortt HE, Garnham PC, *Pre-erythrocytic stage in mammalian malaria parasites*. Nature., 1948. **161**(4082): p. 126.
- 66 Hollingdale MR, Collins WE, Campbell CC, Schwartz AL, *In vitro culture of two populations (dividing and nondividing) of exoerythrocytic parasites of Plasmodium vivax*. Am J Trop Med Hyg. , 1985 **34**(2): p. 216-22.
- 67 Sattabongkot J, Yimamnuaychoke N, Leelaudomlipi S, Rasameesoraj M, Jenwithisuk R et al., *Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites Plasmodium falciparum and P. vivax*. Am J Trop Med Hyg., 2006. **74**(5): p. 708-15.
- 68 Mota MM, Rodriguez A, *Plasmodium yoelii: efficient in vitro invasion and complete development of sporozoites in mouse hepatic cell lines*. Exp Parasitol. , 2000. **96**(4): p. 257-9.
- 69 Prudêncio M, Rodrigues CD, Ataíde R, Mota MM, *Dissecting in vitro host cell infection by Plasmodium sporozoites using flow cytometry*. Cell Microbiol., 2008. **10**(1): p. 218-24.
- 70 Hollingdale MR, Leland P, Schwartz AL, *In vitro cultivation of the exoerythrocytic stage of Plasmodium berghei in a hepatoma cell line*. Am J Trop Med Hyg., 1983 **32**(4): p. 682-4.
- 71 Medica DL, Sinnis P, *Quantitative dynamics of Plasmodium yoelii sporozoite transmission by infected anopheline mosquitoes*. Infect Immun., 2005. **73**(7)(4363-9).
- 72 Protzer U, Maini MK, Knolle PA, *Living in the liver: hepatic infections*. Nat Rev Immunol, 2012. **12**(3): p. 201-13.
- 73 Alberts B, Johnson A, Lewis J, Raff M, Roberts K et al., *Molecular Biology of the Cell*. New York: Garland Science, 2002. **4th edition**.
- 74 Schroder M, Kaufman RJ, *ER stress and the unfolded protein response*. Mutat Res, 2005. **569**(1-2): p. 29-63.

- 75 Schroder M, Kaufman RJ, *The mammalian unfolded protein response*. Annu Rev Biochem, 2005. **74**: p. 739-89.
- 76 Beard NA, Laver DR, Dulhunty AF, *Calsequestrin and the calcium release channel of skeletal and cardiac muscle*. Prog Biophys Mol Biol, 2004. **85**(1): p. 33-69.
- 77 van Meer G, Voelker DR, Feigenson GW, *Membrane lipids: where they are and how they behave*. Nat Rev Mol Cell Biol, 2008. **9**(2): p. 112-24.
- 78 Tabak HF, Braakman I, van der Zand A, *Peroxisome Formation and Maintenance Are Dependent on the Endoplasmic Reticulum*. Annu Rev Biochem, 2013. **82**: p. 723-44.
- 79 Jacquier N, Choudhary V, Mari M, Toulmay A, Reggiori F et al., *Lipid droplets are functionally connected to the endoplasmic reticulum in Saccharomyces cerevisiae*. J Cell Sci. , 2011. **124**(Pt 14): p. 2424-37.
- 80 Weidberg H, Shvets E, Elazar Z, *Biogenesis and cargo selectivity of autophagosomes*. Annu Rev Biochem, 2011. **80**: p. 125-56.
- 81 Levine T, Loewen C, *Inter-organelle membrane contact sites: through a glass, darkly*. Curr Opin Cell Biol, 2006. **18**(4): p. 371-8.
- 82 Kozutsumi Y, Segal M, Normington K, Gething MJ, Sambrook J, *The presence of malformed proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins*. Nature, 1988. **332**(6163): p. 462-4.
- 83 Dorner AJ, Wasley LC, Kaufman RJ, *Increased synthesis of secreted proteins induces expression of glucose regulated proteins in butyrate treated CHO cells*. J Biol Chem. , 1989. **264**(34): p. 20602-7.
- 84 Ron D, Walter P, *Signal integration in the endoplasmic reticulum unfolded protein response*. Nat Rev Mol Cell Biol, 2007. **8**(7): p. 519-29.
- 85 Cao SS, Kaufman RJ, *Unfolded protein response*. Current Biology, 2012. **22**(16): p. R622-626.
- 86 Mori K, *Signalling pathways in the unfolded protein response: development from yeast to mammals*. J Biochem, 2009. **146**(6): p. 743-50.

- 87 Kondo S, Murakami T, Tatsumi K, Ogata M, Kanemoto S et al., *OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes*. *Nat Cell Biol*, 2005. **7**(2): p. 186-94.
- 88 Cox JS, Shamu CE, Walter P, *Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase*. *Cell*, 1993. **73**(6): p. 1197-1206.
- 89 Mori K, Ma W, Gething MJ, Sambrook J, *A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus*. *Cell*, 1993. **74**(4): p. 743-56.
- 90 Korennykh AV, Egea PF, Korostelev AA, Finer-Moore J, Zhang C et al., *The unfolded protein response signals through high-order assembly of Ire1*. *Nature*, 2009. **457**(7230): p. 687-93.
- 91 Papa FR, Zhang C, Shokat K, Walter P, *Bypassing a kinase activity with an ATP-competitive drug*. *Science*, 2003. **302**(5650): p. 1533-7.
- 92 Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K, *XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor*. *Cell*, 2001. **107**: p. 881-891.
- 93 Calton M, Zeng H, Urano F, Till JH, Hubbard SR et al., *IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA*. *Nature*, 2002. **415**(6867): p. 92-6.
- 94 Yamamoto K, Yoshida H, Kokame K, Kaufman RJ, Mori K, *Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II*. *J Biochem*, 2004. **136**(3): p. 343-50.
- 95 Yoshida H, Okada T, Haze K, Yanagi H, Yura T et al., *Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response*. *Mol Cell Biol*, 2001. **21**(4): p. 1239-48.
- 96 Yoshida H, Matsui T, Hosokawa N, Kaufman RJ, Nagata K et al., *A time-dependent phase shift in the mammalian unfolded protein response*. *Dev Cell*, 2003. **4**(2)(265): p. 265-71.

- 97 Lee AH, Iwakoshi NN, Glimcher LH, *XPB-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response*. Mol Cell Biol, 2003. **23**(21): p. 7448-7459.
- 98 Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB et al., *XPB1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation*. Immunity, 2004. **21**(1): p. 81-93.
- 99 Sriburi R, Jackowski S, Mori K, Brewer JW, *XPB1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum*. J Cell Biol, 2004. **167**(1): p. 35-41.
- 100 Tirosh B, Iwakoshi NN, Glimcher LH, Ploegh HL, *Rapid turnover of unspliced Xbp-1 as a factor that modulates the unfolded protein response*. J Biol Chem, 2006. **281**(9): p. 5852-60.
- 101 Yoshida H, Oku M, Suzuki M, Mori K, *pXPB1(U) encoded in XPB1 pre-mRNA negatively regulates unfolded protein response activator pXPB1(S) in mammalian ER stress response*. J Cell Biol, 2006. **172**(4): p. 565-75.
- 102 Hollien J, Weissman JS, *Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response*. Science, 2006. **313**(5783): p. 104-7.
- 103 Hollien J, Lin JH, Li H, Stevens N, Walter P et al., *Regulated Ire1-dependent decay of messenger RNAs in mammalian cells*. J Cell Biol, 2009. **186**(3): p. 323-31.
- 104 Urano F, Wang X, Bertolotti A, Zhang Y, Chung P et al., *Coupling of Stress in the ER to Activation of JNK Protein Kinases by Transmembrane Protein Kinase IRE1*. Science, 2000. **287**(5453): p. 664-666.
- 105 Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K et al., *ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats*. Genes Dev, 2002. **16**(11): p. 1345-55.
- 106 Nguyễn DT, Kebache S, Fazel A, Wong HN, Jenna S et al., *Nck-dependent activation of extracellular signal-regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress*. Mol Biol Cell, 2004. **15**(9): p. 4248-60.

- 107 Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH, *Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression*. Mol Cell Biol, 2006. **26**(8): p. 3071-84.
- 108 Ogata M, Hino S, Saito A, Morikawa K, Kondo S et al., *Autophagy is activated for cell survival after endoplasmic reticulum stress*. Mol Cell Biol, 2006. **26**(24): p. 9220-31.
- 109 Bernales S, McDonald KL, Walter P, *Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response*. PLoS Biol, 2006. **4**(12): p. e423.
- 110 Criollo A, Vicencio JM, Tasdemir E, Maiuri MC, Lavandero S et al., *The Inositol Trisphosphate Receptor in the Control of Autophagy*. Autophagy, 2007. **3**(4): p. 350-3.
- 111 Maiuri MC, Zalckvar E, Kimchi A, Kroemer G, *Self-eating and self-killing: crosstalk between autophagy and apoptosis*. Nat Rev Mol Cell Biol, 2007. **8**(9): p. 741-52.
- 112 Arsham AM, Neufeld TP, *A genetic screen in Drosophila reveals novel cytoprotective functions of the autophagy-lysosome pathway*. PLoS One, 2009. **4**(6): p. e6068.
- 113 Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G et al., *Autophagy suppresses tumorigenesis through elimination of p62*. Cell, 2009. **137**(6): p. 1062-75.
- 114 Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D, *Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response*. Nat Cell Biol., 2000. **2**(6): p. 326-32.
- 115 Harding HP, Zhang Y, Ron D, *Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase*. Nature, 1999. **397**(6716): p. 271-4.
- 116 Kozac M, *Pushing the limits of the scanning mechanism for initiation of translation*. Gene., 2002. **299**(1-2): p. 1-34.
- 117 Scheuner D, Song B, McEwen E, Liu C, Laybutt R et al., *Translational control is required for the unfolded protein response and in vivo glucose homeostasis*. Mol Cell., 2001. **7**(6): p. 1165-76.

- 118 Harding HP, Novoa I, Zhang Y, Zeng H, Wek R et al., *Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells*. Mol Cell. , 2000. **6**(5): p. 1099-108.
- 119 Yaman I, Fernandez J, Liu H, Caprara M, Komar AA et al., *The zipper model of translational control a small upstream ORF is the switch that controls structural remodeling of an mRNA leader*. Cell, 2003. **113**(4): p. 519-31.
- 120 Kroemer G, Mariño G, Levine B, *Autophagy and the integrated stress response*. Mol Cell, 2010. **40**(2): p. 280-93.
- 121 Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN et al., *ER stress triggers apoptosis by activating BH3-only protein Bim*. Cell, 2007. **129**(7): p. 1337-49.
- 122 Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H, *TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death*. The EMBO Journal, 2005. **24**: p. 1243-1255.
- 123 McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ, *Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state*. Mol Cell Biol, 2001. **21**(4): p. 1249-59.
- 124 Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y et al., *CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum*. Genes Dev., 2004. **8**(24): p. 3066-77.
- 125 Tsaytler P, Harding HP, Ron D, Bertolotti A, *Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis*. Science, 2011. **332**(6025): p. 91-4.
- 126 Jousse C, Oyadomari S, Novoa I, Lu P, Zhang Y et al., *Inhibition of a constitutive translation initiation factor 2alpha phosphatase, CReP, promotes survival of stressed cells*. J Cell Biol, 2003. **163**(4): p. 767-75.
- 127 Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD et al., *An Integrated Stress Response Regulates Amino Acid Metabolism and Resistance to Oxidative Stress*. Molecular Cell, 2003. **11**(3): p. 619-633.
- 128 Haze K, Yoshida H, Yanagi H, Yura T, Mori K, *Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress*. Mol Biol Cell, 1999. **10**(11): p. 3787-99.

- 129 Schindler AJ, Schekman R, *In vitro reconstitution of ER-stress induced ATF6 transport in COPII vesicles*. Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17775-80.
- 130 Ye J, Rawson RB, Komuro R, Chen X, Davé UP et al., *ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs*. Mol Cell., 2000. **6**(6): p. 1355-64.
- 131 Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A et al., *ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum*. Cell Struct Funct., 2008. **33**(1): p. 75-89.
- 132 Wu J, Rutkowski DT, Dubois M, Swathirajan J, Saunders T et al., *ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress*. Dev Cell, 2007. **13**(3): p. 351-64.
- 133 Yamamoto K, Sato T, Matsui T, Sato M, Okada T et al., *Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1*. Dev Cell, 2007. **13**(3): p. 365-76.
- 134 Bailey D, O'Hare P, *Transmembrane bZIP transcription factors in ER stress signaling and the unfolded protein response*. Antioxid Redox Signal, 2007. **9**(12): p. 2305-21.
- 135 Zhang K, Shen X, Wu J, Sakaki K, Saunders T et al., *Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response*. Cell, 2006. **124**(3): p. 587-99.
- 136 Vecchi C, Montosi G, Zhang K, Lamberti I, Duncan SA et al., *ER stress controls iron metabolism through induction of hepcidin*. Science, 2009. **325**(5942): p. 877-80.
- 137 Lee MW, Chanda D, Yang J, Oh H, Kim SS et al., *Regulation of hepatic gluconeogenesis by an ER-bound transcription factor, CREBH*. Cell Metab, 2010. **11**(4): p. 331-9.
- 138 Lee JH, Giannikopoulos P, Duncan SA, Wang J, Johansen CT et al., *The transcription factor cyclic AMP-responsive element-binding protein H regulates triglyceride metabolism*. Nat Med, 2011. **17**(7): p. 812-5.

- 139 Brown MS, Goldstein JL, *Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL*. J Lipid Res, 2009. **50 Suppl**: p. S15-27.
- 140 Russell DW, *The enzymes, regulation, and genetics of bile acid synthesis*. Annu Rev Biochem, 2003. **72**: p. 137-74.
- 141 Martin S, Parton RG, *Lipid droplets a unified view of a dynamic organelle*. Nat Rev Mol Cell Biol., 2006. **7(5)**: p. 373-8.
- 142 Miyazaki M, Ntambi J, *Fatty acid desaturation and chain elongation in mammals*. In Biochemistry of lipids, lipoproteins and membranes, D.E. Vance and J.E. Vance, eds (Amsterdam:Elsevier Science), 2008: p. 191-211.
- 143 Krahmer N, Guo Y, Wilfling F, Hilger M, Lingrell S et al., *Phosphatidylcholine synthesis for lipid droplet expansion is mediated by localized activation of CTP:phosphocholine cytidyltransferase*. Cell Metab, 2011. **14(4)**: p. 504-15.
- 144 Fu S, Yang L, Li P, Hofmann O, Dicker L et al., *Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity*. Nature, 2011. **473(7348)**: p. 528-31.
- 145 Colgan SM, Tang D, Werstuck GH, Austin RC, *Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2*. Int J Biochem Cell Biol, 2007. **39(10)**: p. 1843-51.
- 146 Lee JN, Ye J, *Proteolytic activation of sterol regulatory element-binding protein induced by cellular stress through depletion of Insig-1*. J Biol Chem, 2004. **279(43)**: p. 45257-65.
- 147 Lee AH, Scapa EF, Cohen DE, Glimcher LH, *Regulation of hepatic lipogenesis by the transcription factor XBP1*. Science, 2008. **320(5882)**: p. 1492-6.
- 148 Bobrovnikova-Marjon E, Hatzivassiliou G, Grigoriadou C, Romero M, Cavener DR et al., *PERK-dependent regulation of lipogenesis during mouse mammary gland development and adipocyte differentiation*. Proc Natl Acad Sci U S A, 2008. **105(42)**: p. 16314-9.
- 149 Rutkowski DT, Wu J, Back SH, Callaghan MU, Ferris SP et al., *UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators*. Dev Cell, 2008. **15(6)**: p. 829-40.

- 150 Nordlie RC, Foster JD, Lange AJ, *Regulation of Glucose production by the Liver*. Annu Rev Nutr., 1999. **19**: p. 379-406.
- 151 Burchell A, Allan BB, Hume R, *Glucose-6-phosphatase proteins of the endoplasmic reticulum*. Mol Membr Biol., 1994. **11**(4): p. 217-27.
- 152 Harding HP, Novoa I, Zhang Y, Zeng H, Wek R et al., *Regulated translation initiation controls stress-induced gene expression in mammalian cells*. Mol Cell., 2000. **6**(5): p. 1099-108.
- 153 Back SH, Scheuner D, Han J, Song B, Ribick M et al., *Translation attenuation through eIF2 α phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells*. Cell Metab, 2009. **10**(1): p. 13-26.
- 154 Oyadomari S, Harding HP, Zhang Y, Oyadomari M, Ron D, *Dephosphorylation of translation initiation factor 2 α enhances glucose tolerance and attenuates hepatosteatosis in mice*. Cell Metab, 2008. **7**(6): p. 520-32.
- 155 Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH et al., *XBPI controls diverse cell type- and condition-specific transcriptional regulatory networks*. Mol Cell, 2007. **27**(1): p. 53-66.
- 156 Wang Y, Vera L, Fischer WH, Montminy M, *The CREB coactivator CRTC2 links hepatic ER stress and fasting gluconeogenesis*. Nature, 2009. **460**(7254): p. 534-7.
- 157 Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS et al., *NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1 α* . Nature, 2008. **453**(7196): p. 807-11.
- 158 Deng J, Lu PD, Zhang Y, Scheuner D, Kaufman RJ et al., *Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2*. Mol Cell Biol, 2004. **24**(23): p. 10161-8.
- 159 Yamazaki H, Hiramatsu N, Hayakawa K, Tagawa Y, Okamura M et al., *Activation of the Akt-NF-kappaB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response*. J Immunol, 2009. **183**(2): p. 1480-7.

- 160 Li Y, Schwabe RF, DeVries-Seimon T, Yao PM, Gerbod-Giannone MC et al., *Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis*. J Biol Chem, 2005. **280**(23): p. 21763-72.
- 161 Cullinan SB, Diehl JA, *Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway*. Int J Biochem Cell Biol, 2006. **38**(3): p. 317-32.
- 162 Tu BP, Weissman JS, *The FAD- and O2-Dependent Reaction Cycle of Ero1-Mediated Oxidative Protein Folding in the Endoplasmic Reticulum*. Mol Cell., 2002. **10**(5)(983-94): p. 983.
- 163 Martinon F, Chen X, Lee AH, Glimcher LH, *TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages*. Nat Immunol, 2010. **11**(5): p. 411-8.
- 164 Smith JA, Turner MJ, DeLay ML, Klenk EI, Sowders DP et al., *Endoplasmic reticulum stress and the unfolded protein response are linked to synergistic IFN-beta induction via X-box binding protein 1*. Eur J Immunol, 2008. **38**(5): p. 1194-203.
- 165 Bischof LJ, Kao CY, Los FC, Gonzalez MR, Shen Z et al., *Activation of the unfolded protein response is required for defenses against bacterial pore-forming toxin in vivo*. PLoS Pathog, 2008. **4**(10): p. e1000176.
- 166 Richardson CE, Kooistra T, Kim DH, *An essential role for XBP-1 in host protection against immune activation in C. elegans*. Nature, 2010. **463**(7284): p. 1092-5.
- 167 Woo CW, Cui D, Arellano J, Dorweiler B, Harding H et al., *Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signalling*. Nat Cell Biol, 2009. **11**(12): p. 1473-80.
- 168 Bano N, Romano JD, Jayabalasingham B, Coppens I, *Cellular interactions of Plasmodium liver stage with its host mammalian cell*. Int J Parasitol. , 2007. **37**(12): p. 1329-41.
- 169 Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M et al., *A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle*. Mol Biochem Parasitol., 2004. **137**(1): p. 23-33.

- 170 Huang da W, Sherman BT, Lempicki RA, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat Protoc., 2009. **4(1)**: p. 44-57.
- 171 Huang da W, Sherman BT, Lempicki RA, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists*. Nucleic Acids Res., 2009. **37(1)**: p. 1-13.
- 172 Schrag JD, Bergeron JJ, Li Y, Borisova S, Hahn M et al., *The Structure of calnexin, an ER chaperone involved in quality control of protein folding*. Mol Cell., 2001. **8(3)**: p. 633-44.
- 173 Williams DB., *Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum*. J Cell Sci., 2006. **119(Pt 4)**: p. 615-23.
- 174 Sargsyan E, Baryshev M, Szekely L, Sharipo A, Mkrtchian S, *Identification of ERp29, an endoplasmic reticulum luminal protein, as a new member of the thyroglobulin folding complex*. J Biol Chem., 2002. **277(19)**: p. 17009-15.
- 175 Anelli T, Alessio M, Mezghrani A, Simmen T, Talamo F et al., *ERp44, a novel endoplasmic reticulum folding assistant of the thioredoxin family*. EMBO J., 2002. **21(4)**: p. 835-44.
- 176 Knoblach B, Keller BO, Groenendyk J, Aldred S, Zheng J et al., *ERp19 and ERp46, new members of the thioredoxin family of endoplasmic reticulum proteins*. Mol Cell Proteomics, 2003. **2(10)**: p. 1104-19.
- 177 Rini J, Esko J, Varki A, *Glycosyltransferases and Glycan-processing Enzymes*. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 2009.
- 178 Vembar SS, Brodsky JL, *One step at a time: endoplasmic reticulum-associated degradation*. Nat Rev Mol Cell Biol, 2008. **9(12)**: p. 944-57.
- 179 Liang J, Yin C, Doong H, Fang S, Peterhoff C et al., *Characterization of erasin (UBXD2): a new ER protein that promotes ER-associated protein degradation*. J Cell Sci., 2006. **119(Pt 19)**: p. 4011-24.
- 180 Kaufman RJ, Scheuner D, Schröder M, Shen X, Lee K et al., *The unfolded protein response in nutrient sensing and differentiation*. Nat Rev Mol Cell Biol, 2002. **3(6)**: p. 411-21.

- 181 Samali A, Fitzgerald U, Deegan S, Gupta S, *Methods for monitoring endoplasmic reticulum stress and the unfolded protein response*. Int J Cell Biol, 2010. **2010**: p. 830307.
- 182 Ploemen IH, Prudêncio M, Douradinha BG, Ramesar J, Fonager J et al., *Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging*. PLoS One, 2009. **4(11)**: p. e7881.
- 183 Luster AD, *Chemokines--chemotactic cytokines that mediate inflammation*. N Engl J Med., 1998. **338(7)**: p. 436-45.
- 184 Shi C, Pamer EG, *Monocyte recruitment during infection and inflammation*. Nat Rev Immunol., 2011. **1(11)**: p. 762-74.
- 185 Epiphanio S, Mikolajczak SA, Gonçalves LA, Pamplona A, Portugal S et al., *Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver infection*. Cell Host Microbe, 2008. **3(5)**: p. 331-8.
- 186 Stetson DB, Medzhitov R, *Type I interferons in host defense*. Immunity, 2006. **25(3)**: p. 373-81.
- 187 Martinon F, Glimcher LH, *Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum*. Curr Opin Immunol, 2011. **23(1)**: p. 35-40.
- 188 Hasnain SZ, Lourie R, Das I, Chen AC, McGuckin MA, *The interplay between endoplasmic reticulum stress and inflammation*. Immunol Cell Biol, 2012. **90(3)**: p. 260-70.
- 189 Garaigorta U, Chisari FV, *Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation*. Cell Host Microbe, 2009. **6(6)**: p. 513-22.
- 190 Liu J, HuangFu WC, Kumar KG, Qian J, Casey JP et al., *Virus-induced unfolded protein response attenuates antiviral defenses via phosphorylation-dependent degradation of the type I interferon receptor*. Cell Host Microbe, 2009. **5(1)**: p. 72-83.
- 191 Shirey KA, Nhu QM, Yim KC, Roberts ZJ, Teijaro JR et al., *The anti-tumor agent, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), induces IFN-beta-mediated antiviral activity in vitro and in vivo*. J Leukoc Biol, 2011. **89(3)**: p. 351-7.

- 192 Reimold AM, Etkin A, Clauss I, Perkins A, Friend DS et al., *An essential role in liver development for transcription factor XBP-1*. Genes Dev., 2000 **14**(2): p. 152-7.
- 193 Lee J, Sun C, Zhou Y, Lee J, Gokalp D et al., *p38 MAPK-mediated regulation of Xbp1s is crucial for glucose homeostasis*. Nat Med, 2011. **17**(10): p. 1251-60.
- 194 Goma J, Rénia L, Miltgen F, Mazier D, *Iron overload increases hepatic development of Plasmodium yoelii in mice*. Parasitology, 1996. **112** (Pt 2): p. 165-8.
- 195 Stahel E, Mazier D, Guillouzo A, Miltgen F, Landau I et al., *Iron chelators: in vitro inhibitory effect on the liver stage of rodent and human malaria*. Am J Trop Med Hyg., 1988. **9**(3): p. 236-40.
- 196 Heppner DG, Hallaway PE, Kontoghiorghes GJ, Eaton JW, *Antimalarial properties of orally active iron chelators*. Blood., 1988. **72**(1): p. 358-61.
- 197 Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A et al., *Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization*. Science, 2004. **306**(5704): p. 2090-3.
- 198 Bridges JP, Xu Y, Na CL, Wong HR, Weaver TE, *Adaptation and increased susceptibility to infection associated with constitutive expression of misfolded SP-C*. J Cell Biol., 2006. **172**(3): p. 395-407.
- 199 Cheng G, Feng Z, He B., *Herpes simplex virus 1 infection activates the endoplasmic reticulum resident kinase PERK and mediates eIF-2alpha dephosphorylation by the gamma(1)34.5 protein*. J Virol., 2005. **79**(3): p. 1379-88.
- 200 Isler JA, Skalet AH, Alwine JC, *Human cytomegalovirus infection activates and regulates the unfolded protein response*. J Virol. , 2005. **79**(11): p. 6890-9.
- 201 Seimon TA, Kim MJ, Blumenthal A, Koo J, Ehrt S et al., *Induction of ER stress in Macrophages of Tuberculosis Granulomas*. PLoS One., 2010. **5**(9): p. e12772.
- 202 Pillich H, Loose M, Zimmer KP, Chakraborty T., *Activation of the unfolded protein response by Listeria monocytogenes*. Cell Microbiol, 2012. **14**(6): p. 949-64.

- 203 Kawai T, Akira S, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors*. Nat Immunol, 2010. **11**(5): p. 373-84.
- 204 Levrero M, *Viral hepatitis and liver cancer: the case of hepatitis C*. Oncogene, 2006 **25**(27): p. 3834-47.
- 205 Ke PY, Chen SS, *Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro*. J Clin Invest., 2011. **121**(1): p. 37-56.
- 206 Kim I, Xu W, Reed JC, *Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities*. Nat Rev Drug Discov, 2008. **7**(12): p. 1013-30.
- 207 Hall JC, Rosen A, *Type I interferons: crucial participants in disease amplification in autoimmunity*. Nat Rev Rheumatol, 2010. **6**(1): p. 40-9.
- 208 Reiling JH, Clish CB, Carette JE, Varadarajan M, Brummelkamp TR et al., *A haploid genetic screen identifies the major facilitator domain containing 2A (MFSD2A) transporter as a key mediator in the response to tunicamycin*. Proc Natl Acad Sci U S A, 2011. **108**(29): p. 11756-65.
- 209 Randall RE, Goodbourn S, *Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures*. J Gen Virol, 2008. **89**(Pt 1): p. 1-47.
- 210 Bogdan C, Mattner J, Schleicher U, *The role of type I interferons in non viral infections*. Immunol Rev. , 2004. **202**(33-48).
- 211 Stevenson MM, Riley EM, *Innate immunity to malaria*. Nat Rev Immunol, 2004. **4**(3): p. 169-80.
- 212 Liehl P, Mota MM, *Innate recognition of malarial parasites by mammalian hosts*. Int J Parasitol, 2012. **42**(6): p. 557-66.
- 213 Khan ZM, Vanderberg JP, *Role of host cellular response in differential susceptibility of nonimmunized BALB/c mice to Plasmodium berghei and Plasmodium yoelii sporozoites*. Infect Immun., 1991. **59**(8): p. 2529-34.
- 214 van de Sand C, Horstmann S, Schmidt A, Sturm A, Bolte S et al., *The liver stage of Plasmodium berghei inhibits host cell apoptosis*. Molecular Microbiology, 2005. **58**(3): p. 731-742.

- 215 Sommereyns C, Michiels T, *N-glycosylation of murine IFN-beta in a putative receptor-binding region*. J Interferon Cytokine Res., 2006. **26(6)**: p. 406–413.
- 216 Mark DF, Lu SD, Creasey AA, Yamamoto R, Lin LS, *Site-specific mutagenesis of the human fibroblast interferon gene*. Proc Natl Acad Sci U S A. , 1984 **81(18)**: p. 5662-6.
- 217 Colby CB, Inoue M, Thompson M, Tan YH, *Immunologic differentiation between E. coli and CHO cell-derived recombinant and natural human beta-interferons*. J Immunol. , 1984 **133(6)**: p. 3091-5.
- 218 Maheshwari RK, Husain MM, Attallah AM, Friedman RM, *Tunicamycin treatment inhibits the antiviral activity of interferon in mice*. Infect Immun., 1983. **41(1)**: p. 61-6.
- 219 Ho HJ, Huang DY, Ho FM, Lee LT, Lin WW, *Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase expression by endoplasmic reticulum stress*. Cell Signal., 2012. **24(11)**: p. 2166-78.
- 220 Ambrose RL, Mackenzie JM, *West Nile virus differentially modulates the unfolded protein response to facilitate replication and immune evasion*. J Virol. , 2011. **85(6)**: p. 2723-32.
- 221 Minakshi R, Padhan K, Rani M, Khan N, Ahmad F et al., *The SARS Coronavirus 3a protein causes endoplasmic reticulum stress and induces ligand-independent downregulation of the type 1 interferon receptor*. PLoS One, 2009. **4(12)**: p. e8342.
- 222 Tenhunen R, Marver HS, Schmid R., *Microsomal heme oxygenase. Characterization of the enzyme*. J Biol Chem., 1969. **244(23)**: p. 6388-94.
- 223 Kim HP, Pae HO, Back SH, Chung SW, Woo JM et al., *Heme oxygenase-1 comes back to endoplasmic reticulum*. Biochem Biophys Res Commun, 2011. **404(1)**: p. 1-5.
- 224 Briones MR, Tsuji M, Nussenzweig V, *The large difference in infectivity for mice of Plasmodium berghei and Plasmodium yoelii sporozoites cannot be correlated with their ability to enter into hepatocytes*. Mol Biochem Parasitol. , 1996. **77(1)**: p. 7-17.

- 225 Doolan DL, Hoffman SL, *The complexity of protective immunity against liver-stage malaria*. J Immunol., 2000. **165**(3): p. 1453-62.
- 226 Ralph SA, van Dooren GG, Waller RE, Crawford MJ, Fraunholz MJ et al., *Tropical infectious diseases: metabolic maps and functions of the Plasmodium falciparum apicoplast*. Nat Rev Microbiol, 2004. **2**(3): p. 203-16.
- 227 Yu M, Kumar TR, Nkrumah LJ, Coppi A, Retzlaff S, et al., *The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites*. Cell Host Microbe, 2008. **4**(6): p. 567-78.
- 228 Vaughan AM, O'Neill MT, Tarun AS, Camargo N, Phuong TM et al., *Type II fatty acid synthesis is essential only for malaria parasite late liver stage development*. Cell Microbiol, 2009. **11**(3): p. 506-20.
- 229 Pei Y, Tarun AS, Vaughan AM, Herman RW, Soliman JM, et al., *Plasmodium pyruvate dehydrogenase activity is only essential for the parasite's progression from liver infection to blood infection*. Mol Microbiol, 2010. **75**(4): p. 957-71.
- 230 Fu S, Watkins SM, Hotamisligil GS, *The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling*. Cell Metab, 2012. **15**(5): p. 623-34.
- 231 Miyazaki M, Kim YC, Gray-Keller MP, Attie AD, Ntambi JM, *The biosynthesis of hepatic cholesterol esters and triglycerides is impaired in mice with a disruption of the gene for stearoyl-CoA desaturase 1*. J Biol Chem, 2000. **275**(39): p. 30132-8.
- 232 Man WC, Miyazaki M, Chu K, Ntambi J, *Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis*. J Lipid Res, 2006. **47**(9): p. 1928-39.
- 233 Tong L, *Acetyl-coenzyme A carboxylase: crucial metabolic enzyme and attractive target for drug discovery*. Cell Mol Life Sci, 2005. **62**(16): p. 1784-803.
- 234 Graewe S, Stanway RR, Rennenberg A, Heussler VT, *Chronicle of a death foretold: Plasmodium liver stage parasites decide on the fate of the host cell*. FEMS Microbiol Rev., 2012 **6**(1): p. 111-30.
- 235 Jang WG, Kim EJ, Koh JT, *Tunicamycin negatively regulates BMP2-induced osteoblast differentiation through CREBH expression in MC3T3E1 cells*. BMB Rep., 2011. **44**(11): p. 735-40.

- 236 Ashrafiyan H, *Hepcidin: the missing link between hemochromatosis and infections*. Infect Immun., 2003. **71(12)**: p. 6693-700.
- 237 He B, *Viruses, endoplasmic reticulum stress, and interferon responses*. Cell Death Differ, 2006. **13(3)**: p. 393-403.
- 238 Lee SY, Lee MS, Cherla RP, Tesh VL, *Shiga toxin 1 induces apoptosis through the endoplasmic reticulum stress response in human monocytic cells*. Cell Microbiol. , 2008. **10(3)**: p. 770-80.
- 239 Wolfson JJ, M.K., Thorpe CM, Jandhyala DM, Paton JC, Paton AW., *Subtilase cytotoxin activates PERK, IRE1 and ATF6 endoplasmic reticulum stress-signalling pathways*. Cell Microbiol., 2008. **10(9)**: p. 1775-86.
- 240 Maier AG, C.B., Cowman AF, Tilley L., *Malaria parasite proteins that remodel the host erythrocyte*. Nat Rev Microbiol., 009. **7(5)**: p. 341-54.
- 241 Andrews TM, T.J., *Protein synthesis by membrane-bound and free ribosomes of the developing rat cerebral cortex*. Biochem J., 1971. **124(5)**: p. 883-9.
- 242 Snapp, E., *Endoplasmic reticulum biogenesis proliferation and differentiation*. The Biogenesis of Cellular Organelles Mullins C (ed) (New York: Kluwer Academic/Plenum Publishers, 2005: p. 63-95.
- 243 Ozcan, U., et al., *Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes*. Science, 2004. **306(5695)**: p. 457-61.
- 244 Goncalves, L.A., A.M. Vigario, and C. Penha-Goncalves, *Improved isolation of murine hepatocytes for in vitro malaria liver stage studies*. Malar J, 2007. **6**: p. 169.
- 245 Luo, J., et al., *A protocol for rapid generation of recombinant adenoviruses using the AdEasy system*. Nat Protoc, 2007. **2(5)**: p. 1236-47.

