

Characterisation of the unique large Armadillo repeat protein (LARP) in the malaria parasite *Plasmodium berghei*

Jessica Stock B.Sc.

Supervisor: Professor Rita Tewari, PhD

Institution: The University of Nottingham

Date: 15.09.2016

Thesis submitted to the University of Nottingham for the degree of Master of Research.

Abstract

The family of Armadillo(ARM) repeat proteins is known to play key roles in the cellular machinery of eukaryotic cells, including processes like gene expression, cell signalling and cytoskeletal organisation. Their repeating 42 amino acid motif provides a platform for numerous protein-protein interactions. Despite their known importance in eukaryotic cells, ARM repeat proteins remain poorly investigated in Apicomplexan parasites, including the malaria causing *Plasmodium* species. This study characterises the phylum-specific large Armadillo repeat protein (LARP), one of the 10 ARM repeat proteins known in Apicomplexa. For the first time, the localisation of a fluorescent tagged LARP protein was determined in the rodent malaria model system *Plasmodium berghei*. Live cell imaging revealed a predominant cytosolic expression of the protein in every life cycle stage of the parasite. The invasive *P. berghei* stages additionally demonstrate a distinct apical localisation of LARP-GFP, that forms a ring-like structure at the apical end of the cell. Subcellular fractionation assays confirmed the cytosolic expression, but also indicated a partial membrane attachment of LARP, that is possibly enabled through post-translational methylation. The apical expression and membrane attachment of LARP-GFP strongly suggest a localisation of LARP to the apical complex and therefore a connection to the invasion and motility machinery of *P. berghei*. A functional analysis indicated that LARP is likely essential in the asexual blood stages. Despite different approaches in this study, a comprehensive functional analysis through a conditional knockdown system has not yet been achieved. However, the presence of LARP in every *P. berghei* life cycle stage, its distinct properties in invasive parasite stages and apicomplexan-specific nature suggest LARP as a possibly interesting drug-target and highlight the overall importance of ARM repeat proteins in *Plasmodium*.

Acknowledgments

First and foremost, I would like to thank my supervisor Professor Rita Tewari, for giving me the opportunity to do my ERASMUS studentship and Master of Research in her lab and for providing me with this interesting and challenging project, as well as for her support and guidance over the last year. Secondly I would like to acknowledge my second supervisor Professor Fred Sablitzky, for his advice and expertise. Additionally, I would like to thank the lab members (Lorenzo Brusini, Magali Roques and Aline Freville) for their help involving the work with mice, as well as teaching and supporting me over the last year. I would like to extend these acknowledgements to Declan Brady, for his technical assistance and Julie Rogers for keeping the lab running smoothly. Furthermore, I would like to thank Robert Markus for teaching me the essentials of superresolution microscopy. I would like to thank Dominique Soldati-Favre, Damien Jacot and Paco Pino for their help and resources on the Tet-System and their expertise on the subject. Finally, I would like to thank my family and friends, for their support that made the last year possible.

Abbreviations

α	alpha
β	beta
µL	micro-litre
µm	micro-metre
ABP	actin-binding protein
ADP	adenosine diphosphate
APR	apical polar ring
ARM	Armadillo (repeat)
Asex	Asexual blood stages
ATP	adenosine triphosphate
Bp	basepairs
CSP	Circumsporozoite protein
DHFR	dihydrofolate reductase
DIC	differential interference contrast
DNA	Deoxyribonucleic acid
e.g.	for example
F-actin	filamentous actin
g	unit of gravitational acceleration force equal to 9.8 m/s^2

G-actin	globular actin
Gam	Gametocyte
GAP	glideosome associated protein
GFP	green fluorescent protein
GOI	gene of interest
h	hour
HSPG	Heparan sulphate proteoglycans
IBB	importin-beta-binding
IFA	immunofluorescence assay
IMC	inner membrane complex
kb	kilobase (1000 basepairs)
kDa	kilodalton
LARP	large Armadillo repeat protein
LARP-GFP	C-terminally GFP-tagged LARP strain (*P. berghei*)
LARP-KO	*larp* depleted strain (*P. berghei*)
LARP-PTD	LARP promoter swap strain (*P. berghei*)
M	molar
mg	milligrams
MIC	micronemal protein
MJ	moving junction
min	minute

mL	millilitre
mM	millimolar
MSP	Merozoite surface protein
MTIP	Myosin A tail domain interacting protein
MTOC	microtubule-organising centre
nm	nanometre
NLS	nuclear localisation sequence
Ook	Ookinete
PCR	polymerase chain reaction
PFA	Paraformaldehyde
PV	parasitophorous vacuole
RBC	red blood cell
RFP	red fluorescent protein
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-Page	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Shz	Schizont
TCF	transcription factor
TRAP	thrombospondin related adhesive protein
WT	wild type

Table of Contents

1 Introduction 1

1.1 *Plasmodium* life cycle 2

1.1.1 Pre-erythrocytic stage 2

1.1.2 Erythrocytic stages 5

1.1.3 Sexual stages 7

1.1.4 Mosquito stages 8

1.2 The invasive stages of *Plasmodium* berghei 10

1.2.1 Merozoite 10

1.2.2 Ookinete 12

1.2.3 Sporozoite 13

1.3 Invasion and motility of apicomplexan parasites 14

1.3.1 The glideosome complex 14

1.3.1.1 Composition of the glideosome 14

1.3.1.2 The process of gliding motility 17

1.3.2 Invasion process 19

1.3.2.1 Initial host cell contact 20

1.3.2.2 Moving junction and host cell entry 21

1.3.2.3 Host cell egress 23

1.4 Armadillo repeat proteins 24

1.4.1 Armadillo repeat proteins across the animal kingdom 25

1.4.1.1 β-catenin 25

1.4.1.2 Importin-α 27

1.4.1.3 ARM repeat proteins and the cytoskeleton 28

1.4.1.4 ARM repeat proteins and gene expression 29

1.4.1.5 ARM repeat proteins and proteomics 30

1.4.2 Armadillo repeat proteins in Apicomplexa 31

1.4.2.1 PF16 32

1.4.2.2 Armadillo repeat only (ARO) 33

1.4.2.3 PBANKA_113780 35

2 Aims and Objectives 36

3 Material and Methods 38

3.1 Ethics statement 43

3.2 *Plasmodium berghei* strain 43

3.3 Mouse maintenance 43

3.4 Mosquito maintenance 44

3.5 Generation of transgenic parasites 44

3.5.1 Polymerase chain reaction (PCR) for DNA amplification 44

3.5.2 Restriction enzyme digest 45

3.5.3 Ligation and transformation 46

3.5.4 Isolation of plasmid DNA from bacteria 47

3.5.5 Transfection of plasmids into parasites 47

3.6 Genotypic analysis 49

3.6.1 Isolation of asexual blood stages 49

3.6.2 DNA isolation and diagnostic PCR 49

3.7 Analysis of gene expression 50

3.7.1 RNA isolation 50

3.7.2 Quantitative PCR (qRT-PCR) 51

3.8 Proteomics and biochemical analysis 51

3.8.1 Protein preparation 51

3.8.2 Bradford assay 52

3.8.3 Immunoprecipitation and Western Blot 52

3.8.4 Subcellular fractionation 53

3.9 Imaging and microscopy assays 54

3.9.1 Isolation of schizonts, gametocytes and ookinetes 54

3.9.2 Preparation sporozoites 55

3.9.3 Live-cell imaging 55

3.9.4 Immunofluorescence assay 55

3.9.5 Superresolution microscopy 56

4 Results 57

4.1 Expression and Localization of the Large Armadillo repeat Protein (LARP) 57

4.1.1 Genotypic analysis confirms successful integration of LARP-GFP 57

4.1.2 LARP is highly expressed throughout the *Plasmodium berghei* life cycle 59

4.1.3 LARP shows an apical localisation in the invasive stages of *Plasmodium berghei* 61

4.1.4 LARP shows stage-specific developmental profiles during the formation of invasive parasite stages 64

4.1.5 Co-localization of LARP with apical proteins and markers 68

4.2 Biochemical analysis 71

4.2.1 Subcellular fractionation confirms solubility and partial membrane attachment of LARP 71

4.2.2 Interaction of LARP with apical proteins was not validated through proteomic analysis 72

4.3 Functional Analysis 73

4.3.1 LARP is likely to be essential in erythrocytic stages 73

4.3.2 LARP could not be disrupted by the tetracycline-repressible transactivator system 74

4.3.3 Conditional expression of LARP shows no effect on parasite viability and development 76

5 Discussion 81

5.1 Expression and Localisation of LARP 82

5.2 Biochemical properties and interaction partners 87

5.3 Role of LARP in *Plasmodium berghei* 90

5.4 Summary and Outlook 93

6 References 95

7 Appendix 108

List of Figures

Figure 1| *Plasmodium* life cycle 3
Figure 2| The development of the pre-erythrocytic stages of *Plasmodium* 4
Figure 3| The ookinetes journey through the mosquito gut 9
Figure 4|Invasive stages of *Plasmodium* parasites 12
Figure 5| The glideosome in Apicomplexan parasites 15
Figure 6| Time course of events at the apical complex during RBC invasion 20
Figure 7| RBC invasion by *Plasmodium* 22
Figure 8| Structure of Armadillo repeat proteins 25
Figure 9| β-catenin signalling 26
Figure 10| Armadillo repeat proteins in Apicomplexan parasites 31
Figure 11| PF16 is essential for flagella composition and function 32
Figure 12| Role of ARO in *Toxoplasma gondii* 34
Figure 13| Generation of a *Pb*ANKA line expressing LARP-GFP 58
Figure 14| *larp* mRNA expression in different life cycle stages 59
Figure 15| LARP-GFP protein expression throughout the *P. berghei* life cycle ...60
Figure 16| The apical localization of LARP-GFP in invasive stages of *P. berghei* .62
Figure 17| LARP-GFP expression during schizonts and sporozoite development 65
Figure 18| LARP-GFP and MyoA-RFP expression during ookinete development .67
Figure 19| Generating of crossed parasite lines 68
Figure 20| Localization of LARP-GFP in respect to known apical proteins 69
Figure 21| Biochemical analysis of LARP-GFP 71
Figure 22| Schematic representation of the gene knockout strategy. 73
Figure 23| Generation of a tetracycline-repressible transactivator system 75
Figure 24| Genotypic and phenotypic analysis of the promoter swap 77
Figure 25| Phenotypic analysis of the promoter swap 79

List of Tables

Table 1| List of chemicals 38

Table 2| List of solutions 39

Table 3| List of commercial kits 40

Table 4| List of enzymes..... 40

Table 5| List of primers 41

Table 6| List of antibodies..... 42

Table 7| List of materials for microscopy..... 42

1 Introduction

Plasmodium species are malaria causing protozoan parasites belonging to the Subphylum of Apicomplexa. In the present times these parasites still hold a constant threat to humanity, putting almost half of the world population at risk of malaria infection (WHO World Malaria Report, 2015). *Plasmodium* species infect mammals, birds and lizards and are transmitted by the bite of a female mosquito in which the parasite undergoes parts of its life cycle.

The infectious disease malaria is caused by different *Plasmodium* parasites. So far there are five known species causing the disease in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. In 2015 almost 440,000 people died of malaria (WHO World Malaria Report, 2015), ranking the disease amongst the leading causes of death worldwide. Approximately 90% of all fatal malaria outcomes are reported in Africa, habitat of *Plasmodium falciparum*, the most dangerous species of *Plasmodium*.

Plasmodium berghei is a parasite causing malaria in rodents, but shows a strong resemblance to the human parasite *Plasmodium falciparum* in its cell biology and pathogenesis. This rodent species is relatively easy, to genetically manipulate, as all life cycle stages are accessible, either *in vivo* (mice), in blood cultures or in mosquito vectors, and the parasite does not infect humans. Therefore, *Plasmodium berghei* is a perfect model system to study the biology of *Plasmodium* parasites and the pathology of malaria in humans. Even though several malaria drugs and vaccines are known and used to treat malaria patients and prevent infections, none have been shown to be fully effective. Furthermore, increasing drug resistance, for example against the widely used artemisinin (Mbengue et al., 2015), has added another layer of complexity to curing malaria.

1.1 *Plasmodium* life cycle

The *Plasmodium* parasite follows a complex life cycle that can only be completed by the infection of vertebrate host and a mosquito vector, making it complicated to understand its biology. The mammalian infecting *Plasmodium* species are transmitted by the mosquito *Anopheles*. After entering the mammalian host the parasite first invades the liver and eventually reaches the blood stream, where it infects erythrocytes and undergoes asexual development. When the parasite is finally taken up by a feeding mosquito it enters its sexual phase of the life cycle until it is transmitted again into a new mammalian host (Figure 1). In the following sections the different stages of the *Plasmodium* life cycle are explained in more detail.

1.1.1 Pre-erythrocytic stage

When the *Anopheles* mosquito takes its blood meal from a vertebrate it injects *Plasmodium* sporozoites into the hosts skin (Figure 1A). These sporozoites come from the salivary gland of the mosquito, where approximately a hundred are transferred through the saliva (Frischknecht et al., 2004; Vanderberg and Frevert, 2004). The parasites start gliding in a corkscrew motion eventually reaching the blood vessels and breaching the endothelial barrier (Figure 2A), allowing them to enter the blood circulation (Amino et al., 2007; Vanderberg and Frevert, 2004). The sporozoite reaches the liver sinusoid (Figure 2B), a blood vessel with a fenestrated endothelium, which is separated from the hepatocytes of the liver through the space of Disse (Wisse et al., 1985). To reach the liver cells the sporozoite traverses through Kupffer cells located in the sinusoid (Figure 2C), eventually reaching the space of Disse (Pradel and Frevert, 2001; Pradel et al., 2002).

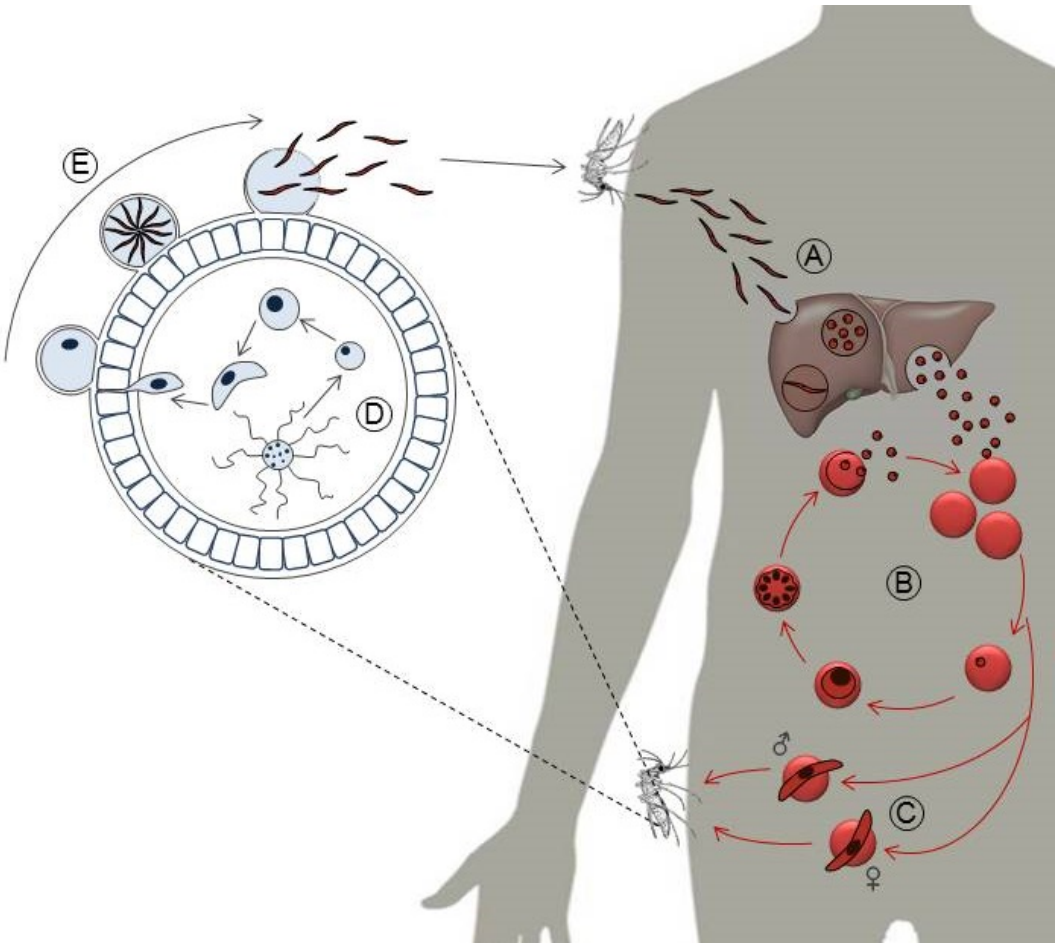


Figure 1| *Plasmodium* life cycle: The *Plasmodium* parasite life cycle alternates between a mammalian host (asexual) and a mosquito vector (sexual and asexual). **(A)** Sporozoites are injected into the mammalian blood stream by a feeding mosquito and eventually reach and invade liver cells. After replication within hepatocytes **(B)**, merozoites are released into the blood stream and invade red blood cells, followed by schizogony that starts an asexual cycle of replication, egress and invasion. **(C)** Eventually, some asexual parasites commit to gametogenesis, forming male and female gametocytes. These are taken in by a feeding mosquito and are **(D)** activated for fertilization by the changing conditions in the mosquito gut. After fertilization, zygotes develop into ookinetes that penetrate the gut wall and round up to form an oocyst **(E)**. Inside the oocyst the parasite undergoes nuclear division and eventually the cell bursts, releasing hundreds of sporozoites into the mosquito's salivary glands. For reference see (Bannister and Sherman, 2009).

Before invading a liver cell and forming a parasitophorous vacuole (PV) the sporozoite traverses through several hepatocytes (Figure 2D), disrupting their membranes and in some cases even causing their cell death (Ishino et al., 2004). The exact reasons and mechanisms behind cell traversal are still not completely

understood, however traversal seems to be necessary for the sporozoite to successfully invade a hepatocyte (Ishino et al., 2005; Kaiser et al., 2004).

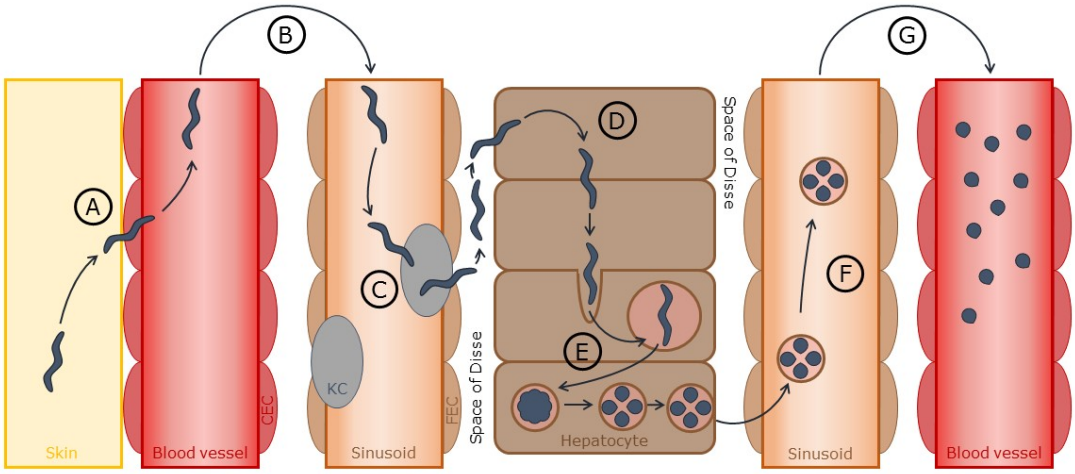


Figure 2| The development of the pre-erythrocytic stages of *Plasmodium*: After being injected into the host's skin, the sporozoites start to glide and breach the endothelial barrier of blood vessels **(A)**. The parasite eventually reaches the liver sinusoid **(B)** and starts traversing through Kupffer cells into the Space of Disse **(C)**. The sporozoites start traversing through several liver cells **(D)** before invading the final hepatocyte. During invasion the parasite forms a parasitophorous vacuole in which it replicates to mature liver stage merozoites **(E)**. The merozoites are transported to the liver sinusoid in merosomes **(F)** and eventually enter the blood stream **(G)** where the merozoites will invade hepatocyte and start asexual replication. Abbreviations: CEC → capillary endothelial cell; KC → Kupffer cells; FEC → fenestrated endothelial cells.

Eventually the parasite switches from its traversal to the invasive state (Figure 2E), to secure its protection from the host's immune system during the replication process. The sulphation level of Heparan sulphate proteoglycans (HSPGs) on the hepatocyte surface indicates which liver cells are suitable for invasion. The sporozoite surface protein Circumsporozoite protein (CSP) binds the glycosaminoglycan chains of HSPG on the host cells surface. Therefore, a higher sulphation of those chains inhibits this binding and the initial attachment of sporozoites to the liver cell (Coppi et al., 2007; Frevert et al., 1993). As soon as the parasite is attached to a suitable host cell, the sporozoite invades the hepatocyte and starts to form a PV, in order to begin its replication process (Meis et al., 1983; Stewart et al., 1986).

24 hours after the invasion, all sporozoite-specific organelles are dissembled from the parasite and the nucleus starts replication. After two days in rodents and six to seven days in primates the parasite (now at the liver schizont stage) is double the size of the original host cell. This indicates the parasites ability to manipulate the hepatocyte to grow without reaching a stage of apoptosis. The parasite starts to differentiate into merozoites, assembling the necessary organelles around the newly formed nuclei (Meis and Verhave, 1988). When the merozoites are fully matured, the parasites induce the death of the host cell and detach from the mother schizont (Sturm et al., 2006; Tarun et al., 2006). Vesicles filled with merozoites (merosomes) leave the liver through the sinusoidal lumen (Figure 2F), passage through the right atrium and ventricle, accumulate in the lungs and break open inside the pulmonary capillaries releasing hundreds of merozoites into the blood stream (Figure 2G) ready to invade erythrocytes (Baer et al., 2007).

These pre-erythrocytic stages of *Plasmodium* are clinically silent, only when the merozoites are released into the blood stream malaria symptoms start to show in the host organism.

1.1.2 Erythrocytic stages

As soon as the merozoites are released into the blood stream they are vulnerable to an immune attack from the host organism. Therefore, the parasite has to recognise, attach to and invade a suitable red blood cell as soon as possible (Figure 1B). This process takes less than 60 seconds to secure an immediate protection of the parasite cell inside the host cell (reviewed by Cowman and Crabb, 2006). The merozoite can distinguish erythrocytes from other cell types through host cell receptors on their surface. The initial attachment of a parasite to the host cell can happen at any point of the merozoite surface. The parasite then undergoes

a reorientation, eventually pointing its apical end towards the host cell membrane (Bannister and Dluzewski, 1990).

Adhesins secreted from the parasite's apical end and host cell receptors form a tight junction between merozoite and erythrocyte membrane. This so-called moving junction (MJ) provides a stable connection between the two cells. The MJ then moves from the apical to the posterior end, forming a ring around the parasite, until the host cell membrane is enclosing the parasite (reviewed by Shen and Sibley, 2012). This process involves the forward movement of the merozoite itself, that is driven by the force of the actin-myosin motor (described in more detail in chapter 1.3.1.1) (Keeley and Soldati, 2004). Once inside the host cell the parasite forms a parasitophorous vacuole, sealing it from the host cell cytoplasm and creating a hospitable environment for parasite replication.

After the invasion process the merozoite loses its invasive organelles (rhoptries, micronemes and dense granules). This stage of asexual development in the erythrocyte is called a ring stage, as the invaded blood cell has a disk-like shape with a thinner centre and, after Giemsa staining, resembles the form of a ring. The parasite starts feeding from the host cells cytoplasm to promote its growth and eventual replication. The haemoglobin taken in by the parasite is proteolytically degraded and the residual haem stored as haemozoin in the large food vacuole within the forming schizont. The intake of nutrients allows the parasite to grow and reach the trophozoite stage. The parasite eventually starts nuclear division and undergoes schizogony. Each nucleus enters a merozoite bud and assembles all necessary organelles near it. After complete nuclear division (now schizont stage) and full maturation of the merozoites the parasite detaches from the mother schizont, ready to egress (reviewed by Bannister and Sherman, 2009).

The parasites egress is induced by different factors: the growing schizont causes a constant increase of the intracellular volume and therfore puts the host

cell membrane and cytoskeleton under pressure and weakens their stability. Additionally, a series of chemical reactions lead to biochemical changes within the host cell, further destabilizing cytoskeleton and membrane eventually causing the cell to burst (Salmon et al., 2001; Wickham et al., 2003). This explosive rupture disperses the non-motile merozoites into the blood stream, allowing them to start another round of invasion and replication (Figure 1B) (Glushakova et al., 2005).

1.1.3 Sexual stages

During the life cycle of asexual blood stages, a subpopulation of intracellular parasites commits to sexual development and undergoes gametogenesis (Figure 1C). The commitment of a parasite to undergo sexual development has to take place before the final stage of schizogony (Eksi et al., 2005; Pace et al., 2006), as either all or no merozoites of one schizont develop into gametocytes. Until recently the factors and mechanisms leading to gametogenesis were unknown. New research however, identified the transcription factor PbAP2-G to be an important regulator of gametogenesis in *Plasmodium berghei*. PbAP2-G depleted parasites are unable to commit to gametogenesis and show an increase in asexual growth, as well as a downregulation in major gametocyte-specific genes (Sinha et al., 2014). Recent studies additionally provide evidence that the silencer Hda2 and the heterochromatin protein HP1 regulate the expression of AP2-G and therefore control sexual commitment of asexual *Plasmodium* blood stages (Brancucci et al., 2014; Coleman et al., 2014).

Gametocytes present in the blood are taken in by a feeding mosquito and are activated in the mosquito gut. A drop in temperature to 20°C, a rise in the pH value and the presence of xanthurenic acid, a metabolic intermediate in the mosquito gut, are factors triggering this process (Billker et al., 1998, 2004). Upon activation male gametes undergo three rounds of DNA replication, which takes approximately 15 minutes. During this process the male gamete assembles eight

flagella at its surface (exflagallation) that eventually fertilise a female gamete (Guttery et al., 2012). Female gametocytes do not undergo DNA replication. After the female parasite exits the red blood cell a male gamete attaches and induces cellular, then nuclear fusion. After fertilization, DNA replication and meiosis, a zygote is formed from the fertilized cell (Figure 1D) (Janse et al., 1986).

1.1.4 Mosquito stages

After its formation the spherical zygote starts developing into an ookinete (Figure 1D). Typically, during a feed, a few hundred gametocytes are taken in by the mosquito. From this less than a hundred ookinetes are formed and out of these even fewer make it to the gut wall to form oocysts. However, these few oocysts produce thousands of sporozoites and secure successful transmission to the vertebrate host (Alavi et al., 2003). These parasite losses can be explained with the many mosquito defence mechanisms the parasite has to face (reviewed by Osta et al., 2004; Severo and Levashina, 2014). When the parasite is recognised as a foreign pathogen, the mosquito immune system is activated. This leads to three major responses, which take place within the midgut epithelium. Firstly, penetrated gut cells induce the expression of nitric oxide synthase (NOS) which leads to high levels of NOs. This hostile environment forces the cell into apoptosis and the ookinete to traverse quickly enough into a new cell, before dying itself (Baton and Ranford-Cartwright, 2004; Han and Barillas-Mury, 2002; Han et al., 2000). However, the two processes most accountable for the enormous parasite loss within the mosquito are melanisation (reviewed by Christensen et al., 2005) and intra-epithelial lysis (Blandin et al., 2004), that are induced by the mosquito complement-like system.

Therefore, the ookinete quickly traverses through the mosquito midgut and settles at the basal lamina to form an oocyst. It first has to pass through the peritrophic matrix (Figure 3A), a matrix mostly containing chitin cross-linked by

peritrophins that surrounds the blood meal (Tellam et al., 1999). The ookinete squeezes through an enzymatically digested hole and escapes the blood meal bolus, into the mosquito gut (Huber et al., 1991; Langer and Vinetz, 2001). The exact components involved in this process are not known. However, MAOP (membrane attack ookinete proteins) a micronemal protein containing a perforin-like membrane attack complex domain, is a promising candidate (Kadota et al., 2004).

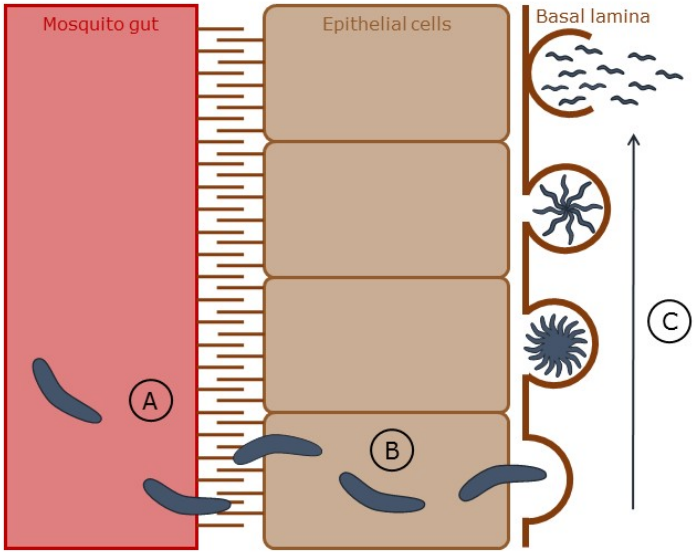


Figure 3| The ookinetes journey through the mosquito gut: After the zygote has fully developed into an ookinete, the parasite penetrates the peritrophic matrix **(A)** and starts to traverse through the epithelial cell layer **(B)** until it reaches the basal lamina. The ookinete rounds up to form an extracellular oocyst and starts mitotic division to form hundreds of sporozoites. Mature sporozoites escape from the oocyst **(C)** and invade the salivary glands.

The ookinete then penetrates the midgut epithelium of the mosquito gut and starts traversing through the epithelial cell (Figure 3B) (Kariu et al., 2006; Zieler et al., 1999). During this process the parasite does not form a parasitophorous vacuole, which distinguishes it from other invasive *Plasmodium* stages. As soon as the parasite reaches the basal side of the epithelium the ookinete switches from traversal to sessile mode and rounds up at the basal side ready for oocyst formation (Figure 1E) (Schneider and Shahabuddin, 2000; Warburg and Schneider, 1993). Within the oocyst, mitotic division starts, strictly

separating karyokinesis and cytokinesis (Nacer et al., 2008; Sinden et al., 2004). The oocyst plasma membrane creates internal invaginations to form lobes of cytoplasm, the so called sporoblasts. Inside, the sporozoite starts to form. While MTOCs (Microtubule-organizing centre) lead to the formation of an apical complex and help positioning the nucleus within the sporoblast (Nacer et al., 2008; Vanderberg and Rhodin, 1967). As soon as the sporozoites are fully formed they can escape through small holes within the oocyst capsule wall caused by growth and proteases (Aly and Matuschewski, 2005; Sinden and Garnham, 1973). The sporozoites leave the oocyst and make their way to the salivary glands, ready for transmission into the next vertebrate host (Figure 3C).

1.2 The invasive stages of *Plasmodium* berghei

During the three developmental stages of the *Plasmodium berghei* life cycle the parasites develops distinct structural features and organelles that allow it to invade different host cells and move across a substrate surface using gliding motility. These invasive stages of *Plasmodium* exhibit a form of gliding movement, that is unique to this phylum. In the following sections these invasive parasite stages are described in more detail.

1.2.1 Merozoite

The merozoite is the smallest invasive stage (1 µm in diametre) of the *Plasmodium* life cycle (Figure 4A). It is the invasive stage during the asexual development in the blood cells. However, unlike other invasive stages the merozoite is non-motile and gets distributed in the blood stream through the outburst from a former erythrocytic host cell (Bannister and Sherman, 2009).

During this stage the parasite has an oval form with a prominence at the front (anterior end), where the apical polar ring (APR) is located (Bannister et al.,

2000). The APR serves as one of three microtubule-organizing centres (MTOCs) to whom three or four subpellicular microtubules are attached and running along the inner membrane complex for approximately 2/3 of the parasite's length. Together with the rhoptries and micronemes the APR forms the apical complex, a structural feature specific for apicomplexan parasites that is further discussed in section 1.3.2 (Bannister and Mitchell, 1995; Nichols and Chiappino, 1987; Russell and Burns, 1984). The merozoite's plasma membrane is covered with a thick bristly coat of surface proteins and filaments that is shed after entry into the host cell (Bannister et al., 1986). Recent studies suggest that the parasite only loses specific surface proteins that are involved in the initial steps of invasion (e.g. N-terminal MSP1, MSP7, MSP3, SERA4 and SERA5). Surface proteins, important during the invasion process (e.g. MSP2), on the other hand, are kept at the surface and degraded after successful invasion. Additionally, these studies identified surface proteins that were internalized rather than degraded (e.g. MSP4) and are likely to play a role in post invasion processes (Boyle et al., 2014). Underneath the coated plasma membrane lies the inner membrane complex (IMC) that is composed of an inner and an outer membrane, to which the invasion and motility machinery (glideosome, section 1.3.1.1) is anchored (reviewed by Morrissette, 2015; Morrissette and Sibley, 2002).

Additional to these structural features the merozoite contains a single nucleus, a mitochondrion, an apicoplast (chloroplast-like organelle) and several ribosomes, all organelles essential for the parasite survival and development of the following life cycle stages.

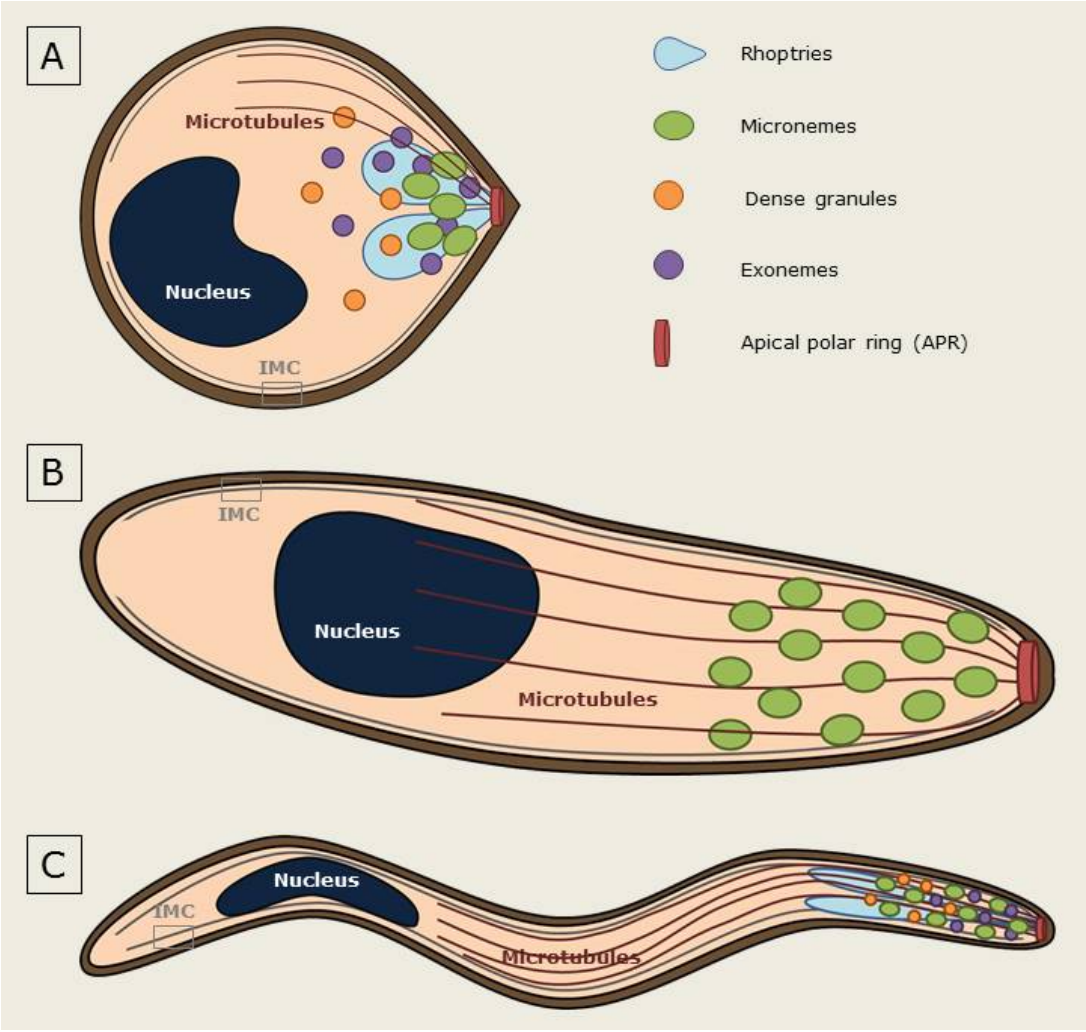


Figure 4|Invasive stages of *Plasmodium* parasites: *Plasmodium* forms three different invasive stages during its life cycle. Merozoites **(A)** are non-motile and invade red blood cells, ookinetes **(B)** are motile and penetrate the mosquito gut wall and sporozoites **(C)** are the motile parasites that get transmitted into the vertebrate host and invade liver cells. All invasive stages share a common structural feature: the apical complex. Every organelle of the apical complex is essential for gliding motility and provides the necessary molecules for this unique form of movement. The complex consists of an apical ring, micronemes, rhoptries, dense granules and the still widely uncharacterised exonemes. The latter three cannot be found in ookinetes, as these are extracellular parasite forms. Further characteristic features of these stages are the inner membrane complex (IMC) and the subpellicular microtubules that run underneath the inner membrane of the IMC. Together these structures provide stability for the parasite. For reference see (Baum et al., 2008)

1.2.2 Ookinete

After the fertilization of female gametes in the mosquito gut a zygote differentiates into a motile and invasive ookinete, which eventually invades the

mosquito gut wall. It contains the same essential organelles as a merozoite, however is much larger in size (10 µm long) and has an elongated, banana-like shape. Therefore, its cytoskeleton contains more microtubules (~60) running underneath the IMC along the first two thirds of the parasite's body (Figure 4B) (Morrissette, 2015; Morrissette and Sibley, 2002; Sinden et al., 1985).

Unlike the merozoite or sporozoite, the ookinete is an extracellular parasite stage and therefore does not need to invade host cells, but only penetrate the mosquito gut wall. This difference in biology is reflected in its structural features: the parasite has a reduced apical complex and for example completely lacks rhoptry organelles (Patra and Vinetz, 2012), as these stages do not need to form a parasitophorous vacuole.

1.2.3 Sporozoite

The sporozoite is the only *Plasmodium* life cycle stage present in the mosquito vector and vertebrate host. This feature makes it unique amongst the invasive stages. Its structural attributes and organelle content are quite similar to these of a merozoite. However, the sporozoite is much longer (10 µm long, 1 µm wide) and slightly curved (Bannister and Sherman, 2009). Starting at the apical polar ring several subpellicular microtubules form a spiral-cage like cytoskeleton right underneath the IMC, similar to the other invasive life cycle stages (Figure 4C) (Morrissette, 2015; Morrissette and Sibley, 2002).

1.3 Invasion and motility of apicomplexan parasites

1.3.1 The glideosome complex

Unlike many other single celled organisms, the motility of apicomplexan parasites is not reliant on an organelle, like for example a flagellum, but rather it is dependent on the interaction with a substrate. The core of this extraordinary gliding machinery is an actomyosin-motor located in the apical part of the parasite that interacts with host-cell binding surface proteins. The complex of these proteins in Apicomplexan parasites is called the glideosome and provides the mechanical force for the parasite to move along and invade host cells (Figure 5) (Boucher and Bosch, 2015; Cowman et al., 2012).

1.3.1.1 Composition of the glideosome

The core molecules of the glideosome are actin and myosin. Myosin A (MyoA) is a member of the unconventional class XIV of myosins and is restricted to Apicomplexa (Foth et al., 2006; Heintzelman and Schwartzman, 1997; Meissner et al., 2002). During the development of invasive parasite stages MyoA forms a pre-complex with other components of the glideosome (Figure 5A). MyoA recruits the myosin light chain PfMTIP (Myosin A tail domain interacting protein) (Green et al., 2006) through an IQ motif (calmodulin binding motif) in its neck domain and binds GAP45 (glideosome associated protein) in the cytosol, before being anchored to the IMC by GAP50 (Bergman et al., 2003; Herm-Götz et al., 2002; Johnson et al., 2007; Kappe et al., 1999). Until now the role of GAP45 in this complex is not completely understood (Gaskins et al., 2004). However, it is known that GAP45 forms a heterotetramer with GAP50 in the outer membrane of the IMC through which the complex is anchored. This interaction is stimulated by the reversible palmitoylation of GAP45 (Green et al., 2008; Rees-Channer et al., 2006), leading

to the hypothesis that GAP45 could regulate the motor function by controlling the anchorage of the myosin-complex to the IMC (Gilk et al., 2009).

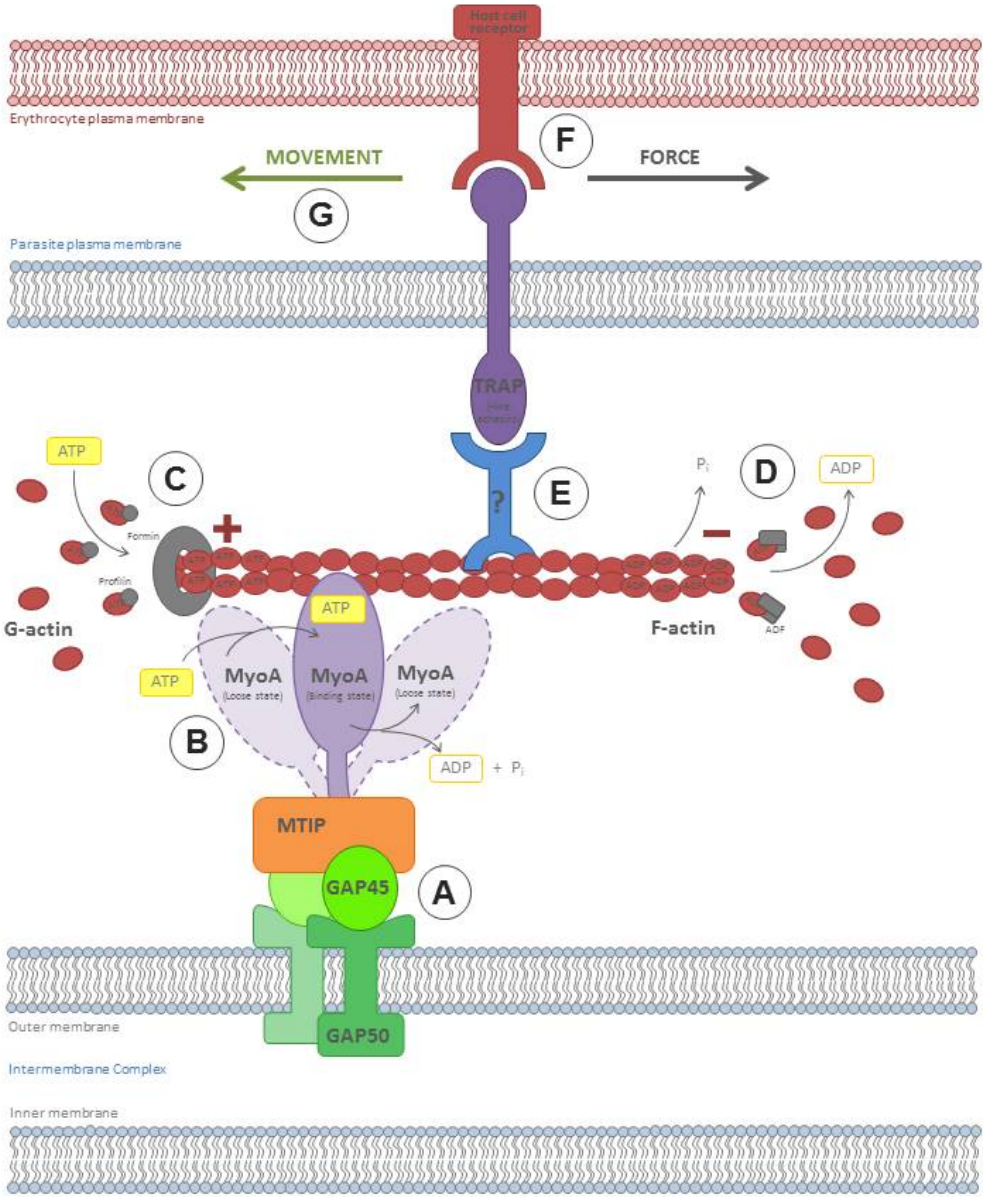


Figure 5| The glideosome in Apicomplexan parasites: The glideosomal complex provides the necessary force for the gliding motility of apicomplexan parasites. MyoA transforms chemical energy (ATP) into mechanical movement. MyoA is anchored to the outer membrane of the IMC through a heterotetramer formed by GAP50 and GAP45 **(A)**. Through the hydrolysis of ATP to ADP MyoA can bind to an F-actin filament and relocate it during a power stroke **(B)**. F-actin filaments rapidly polymerise and depolymerise, taking in ATP-G-actin monomer at the (+)-end **(C)** and releasing ADP-G-actin monomers at the (-)-end **(D)** with the help of actin-binding proteins. Through a so far unknown connector the actin filaments are connected to the cytosolic domain of TRAP-like adhesins **(E)** in the parasites plasma membrane. The adhesins are interacting with receptors at the host-cells surface **(F)** and thereby transferring the force created by the MyoA power stroke into parasite movement across the host cell surface **(G)**. For reference see (Keeley and Soldati, 2004).

As MyoA is anchored and immobilized in the IMC, its direct interaction partner actin has to form mobile filaments to transfer the force of myosin into parasite movement. Interestingly, the major part of actin in apicomplexan parasites is present in its monomeric form rather than polymerized, indicating a tightly regulated and dynamic polymerization of actin molecules (Figure 5C and D) (Dobrowolski et al., 1997). Compared to actin in other species, such as rabbit skeletal actin that forms elongated microfilaments with up to 1000 protomers, apicomplexan actin is rather short and only reaches 30 to 70 protomers (Schmitz et al., 2005). Therefore, the myosin head domains need to be located close together, to allow actin the interaction with more than one myosin molecule at once (Sahoo et al., 2006; Schüler et al., 2005a).

To connect the glideosome to the parasite surface and transform mechanical force into gliding motility actin is indirectly interacting with adhesins (Figure 5E). These proteins are secreted onto the parasite surface by the organelles of the apical complex and interact with receptors on the host cell surface (Figure 5F). These adhesins vary between the life cycle stages and are therefore specific for the type of host cell they are recognizing (Cowman and Crabb, 2006).

The best characterised and studied adhesin is the thrombospondin related adhesive protein (TRAP in *Plasmodium* species (Heiss et al., 2008) and TgMIC2 in *Toxoplasma* (Huynh et al., 2003)), which is expressed on the surface of sporozoites (Sultan et al., 1997). TRAP is a type I integral membrane protein and binds sulphated glycosaminoglycans on liver cells. This adhesive function is mediated by the thrombospondin repeat (TSR) domain, a domain found in all five known members of the TRAP family acting as adhesins in *Plasmodium* parasites (Müller et al., 1993; Tan et al., 2002): the mentioned TRAP and the TRAP-like protein (TLP) in sporozoites (Heiss et al., 2008; Lacroix et al., 2008), the Circumsporozoite- and TRAP-related protein (CTRP) in ookinetes (Vlachou et al., 2006) and the Merozoite TRAP (MTRAP) (Baum et al., 2006a) and *Plasmodium*

thrombospondin related apical merozoite protein (PTRAMP) in merozoites (Thompson et al., 2004). Through their extracellular domain these proteins interact with receptors on the host cell surface and with their cytosolic tail-domain adhesins are able to interact with the parasite glideosome. Until recently, the enzyme aldolase was thought to build the connection between actin and the adhesins (Buscaglia et al., 2003; Jewett and Sibley, 2003), as this protein was shown to interact with actin as well as the cytosolic domain of TRAP proteins. However, new studies have refuted this theory. In 2009 Starnes et al., generated a conditional knockdown of TgALD (*Toxoplasma gondii* aldolase) in *Toxoplasma gondii*, which was severely impaired in its invasion and gliding motility. However, by complementing this knockdown line with an ALD protein lacking the adhesin binding domain, the phenotype was rescued and the cells fully motile again. These experiments indicate that aldolase is essential for the parasites motility because of its enzymatic activity rather than its function as a bridge between actin and the adhesins (Starnes et al., 2009). Therefore, until today the molecule or molecule complex connecting the glideosomal motor to the surface adhesins remains unknown.

1.3.1.2 The process of gliding motility

The glideosome provides the necessary force for the gliding related processes within the parasite through its actomyosin core complex. The parasite follows three distinct patterns of movement that have been identified *in vitro*. The circular gliding motility and a rotation-driven helical gliding behaviour result in a productive forward translocation of the parasite. When anchored at the posterior pole the parasite follows into a vertical, non-productive twirling behaviour (Håkansson et al., 1999). *In vivo* however, the parasites show an irregular corkscrew-like movement with different speeds and patterns depending on the life cycle stage and the environment (Frischknecht et al., 2004; Vlachou et al., 2004).

The force enabling the parasite to move forwards is provided by the glideosome and likely to be controlled by actin polymerization. As mentioned above the formation of F-actin is a tightly regulated and highly dynamic process. As actin is mostly present in its globular form within the parasites its polymerization could be an initiating event to trigger myosin binding and the conversion of chemical into mechanical energy (Baum et al., 2006b; Gordon and Sibley, 2005). The exact signalling cascades controlling the assembly of F-actin are mostly unknown; however, the overall process is quite well understood: globular actin-monomers (G-actin) take up ATP which allows them to bind to the (+)-end of a forming F-actin filament (Figure 5C). This reaction is regulated by several actin-binding proteins (ABP). While the typical Arp2/3 complex (an actin-cytoskeleton regulating complex in other eukaryotic cells) is not present in Apicomplexa, there are two formins (ABPs involved in the polymerization of actin) conserved amongst apicomplexan parasites. Together with profilin, an ABP involved in the actin skeleton turnover, these formins are known to initiate the rapid formation of F-actin filaments (Afonina et al., 2016; Baum et al., 2008). Once bound in the actin filaments the individual subunits hydrolyse their bound ATP to ADP and a phosphate. This reaction lowers the affinity between the actin subunits and with assistance from actin-binding protein ADF (actin depolymerising factor) ADP-actin-monomers leave the filament at the (-)-end (Figure 5D) (Allen et al., 1997; Schüler et al., 2005b; Van Troys et al., 2008). After exchanging ADP for a new ATP molecule, ATP-G-actin is ready to be taken up into the actin-filament again, creating a dynamic and balanced assembly and disassembly of actin filaments.

The polymerised F-actin filament can be bound by Myosin A. This interaction that is essential for generating the necessary force to move the parasite forward. The loose Myosin A head domain takes in an ATP molecule and binds to F-actin. By cleaving ATP into ADP and phosphate the myosin head can perform a

power stroke, relocating the actin filaments and pulling the whole cell towards the bound host cell or substrate. After the power stroke myosin takes in a new ATP molecule, is able to detach itself from the actin filaments and is ready for binding the relocated filaments again to perform another power stroke (Figure 5B) (Daher and Soldati-Favre, 2009).

By creating a force that pushes the actin filaments and the adhesin molecules in the direction of the Myosin A power stroke this process pushes the parasite in the opposite direction, allowing it to move forward, along the substrate surface (Figure 5G).

1.3.2 Invasion process

The invasion by apicomplexan parasites is a complex and rapid process that still holds many unanswered questions. During this process the parasite uses the apical and the glideosomal complex to actively invade its host cell. The apical complex is comprised of four different organelles: micronemes, rhoptries, dense granules and exonemes. All of these organelles secrete proteins and lipids during different stages of the invasion process and thereby allow sporozoites (hepatocytes) and merozoites (erythrocytes) to actively invade host cells using the actomyosin motor imbedded in the glideosome (reviewed by Cowman et al., 2012). The mechanisms of red blood cell invasion are much better understood than the invasion by sporozoites, as these life cycle stages are much easier to study. However, as merozoites and sporozoites have a similar physiological structure, organelle content and both form a PV, it is likely that these two processes follow a similar pattern. Ookinetes are not actively invading host cells, but only penetrating the mosquito gut wall to embed as an extracellular oocyst. Therefore, the next sections will focus on the mechanisms of red blood cell invasion.

1.3.2.1 Initial host cell contact

After merozoites have egressed from the liver cells the potassium levels within the parasite cell drastically decrease, leading to a release of calcium. The calcium signalling cascade leads to the secretion of adhesins and invasins from the micronemes (Figure 6) onto the host cell surface (Singh et al., 2010; Srinivasan et al., 2011; Treeck et al., 2009). These surface proteins allow long-distance and low affinity interactions with erythrocytes in the blood stream. The initial contact between parasite and host cell can take place at any point of the merozoite surface and is followed by a reorientation of the parasite to bring the apical end towards the host cell membrane (Figure 7A) (Bannister and Dluzewski, 1990). There are two groups of proteins on the merozoite surface, which are involved in these

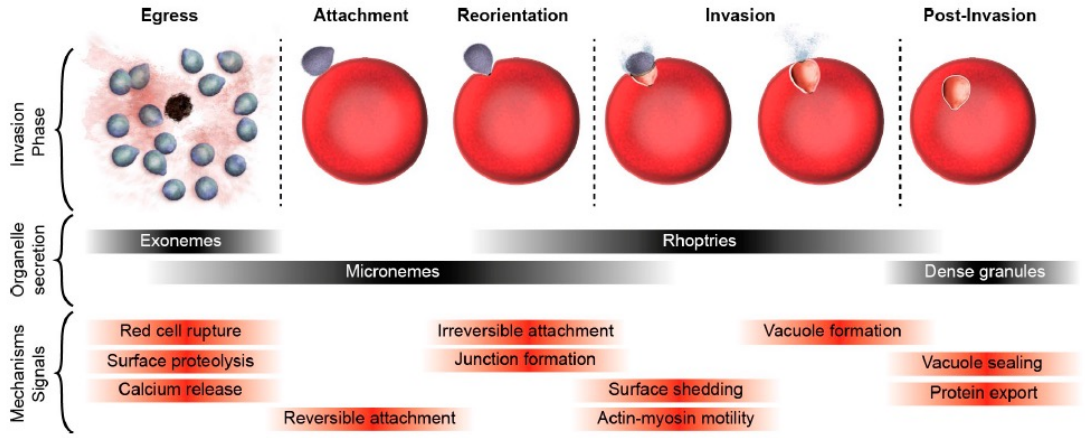


Figure 6| Time course of events at the apical complex during RBC invasion: During host cell invasion the order of organelle secretion is of high importance for a successful invasion process. In mature schizonts exonemes secrete their content to induce host cell egress. Upon the attachment to a new host cell micronemes secrete their proteins to form a tight junction with the host cell surface. The rhoptries follow immediately with the release of their content to drive invasion and the formation of a PV. The last organelles to secrete their content are dense granules, whose molecules are essential for providing a hospitable environment for the parasite. Image adapted from (Cowman et al., 2012)

processes. Firstly, GPI-anchored proteins, which are surface proteins attached to the plasma membrane through glycosylphosphatidylinositol (Sanders et al., 2005). Most of these proteins belong to the MSP family, with MSP-1 being the

most abundant and well characterised protein. It is functionally conserved throughout apicomplexan parasites and has been shown to be essential for parasite invasion. It binds directly to the surface Band 3, an anion transport protein regulating anion exchange between plasmamembranes (Gerold et al., 1996; Holder, 1988). This bond between host cell and parasites is one of many connections that secure a stable attachment of the parasite. Besides GPI-anchors there are many other proteins associated to the membrane through indirect linkage, which are involved in parasite attachment, such as erythrocyte binding antigens (EBA) or reticulocyte-binding-like proteins (Adams et al., 1992; Haynes et al., 1988).

Two of the most well-studied micronemal proteins that are secreted during host-cell attachment are AMA1 (Peterson et al., 1989) and TRAP (Robson et al., 1988). These two proteins provide a stable connection between host cell and parasite and are essential for the formation of a moving junction and the host cell entry.

1.3.2.2 Moving junction and host cell entry

After reorientation and secure attachment of the parasite, the rhoptries start to secrete their proteins. Rhoptries are pear-shaped organelles that always come in pairs and are bound to the plasma membrane. Rhoptries are composed of two subcompartments: the apical duct or neck and the bulb (Bannister et al., 2000; Cowman and Crabb, 2002). Rhoptry neck proteins (RONs) are highly conserved amongst apicomplexan species (Oakes et al., 2013) and are mainly involved in host cell adhesin before and after invasion. Together with the micronemal protein AMA1 the RONs form a complex, the so-called moving junction (MJ) (Figure 7B) (Besteiro et al., 2011; Shen and Sibley, 2012). This complex forms a ring-like structure around invading merozoites that provides a tight molecular connection between parasite and host cell membrane. While the MJ

moves from the apical to the posterior end of the parasite, it remains static at the host cell membrane. This causes the parasite to move into the erythrocyte and the host cell membrane wrapping around the merozoite (Figure 7A). The MJ movement is driven by the actomyosin motor (Keeley and Soldati, 2004), however the core functional components of the MJ and their connection to the motility machinery inside the parasite remain widely unknown.

While proceeding further into the host cell the parasite also releases the rhoptry bulb proteins and lipids which are responsible for the formation of a PV. The parasitophorous vacuole provides shelter from the host cell cytoplasm and

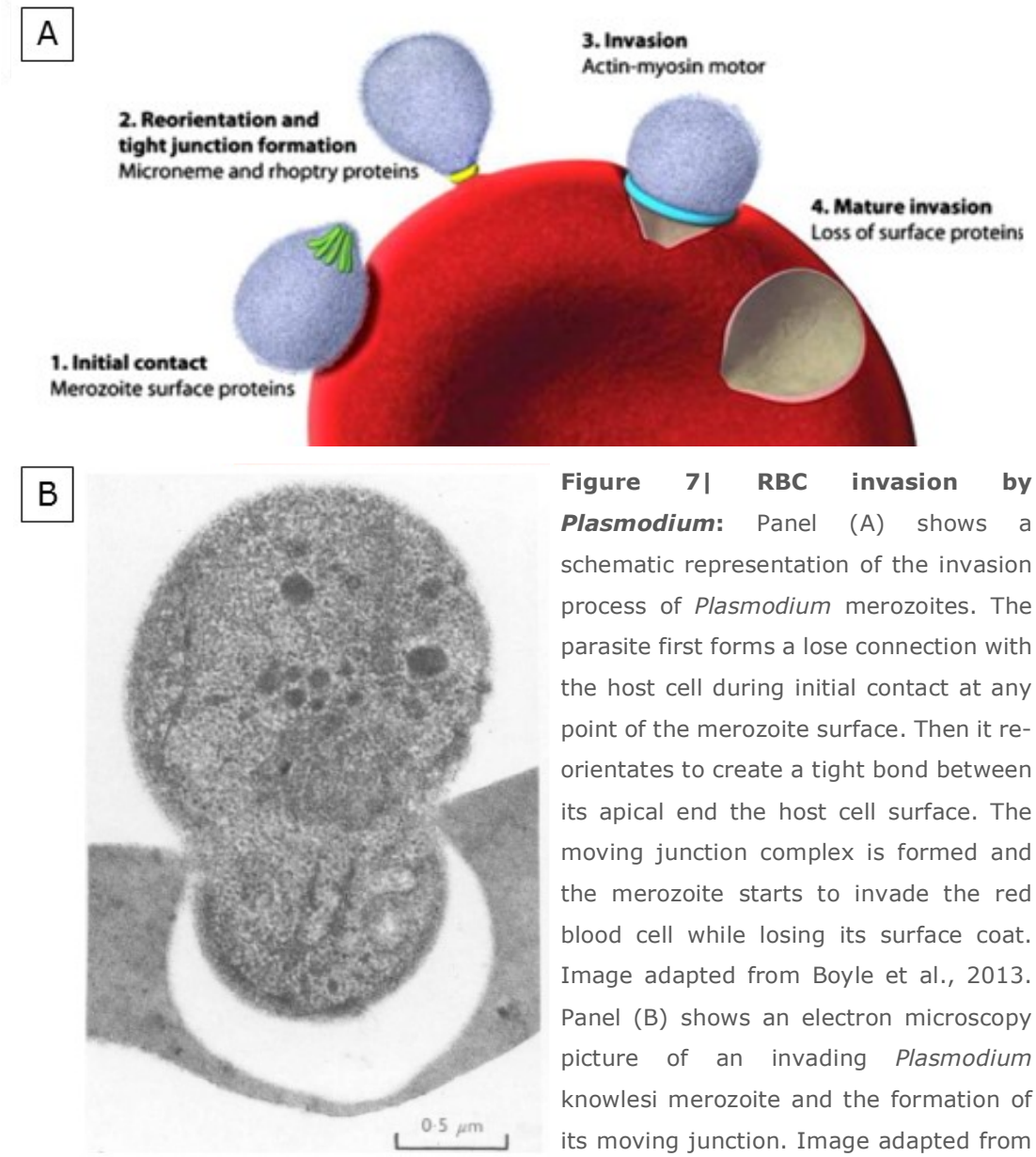


Figure 7| RBC invasion by *Plasmodium*: Panel (A) shows a schematic representation of the invasion process of *Plasmodium* merozoites. The parasite first forms a lose connection with the host cell during initial contact at any point of the merozoite surface. Then it re-orientates to create a tight bond between its apical end the host cell surface. The moving junction complex is formed and the merozoite starts to invade the red blood cell while losing its surface coat. Image adapted from Boyle et al., 2013. Panel (B) shows an electron microscopy picture of an invading *Plasmodium* knowlesi merozoite and the formation of its moving junction. Image adapted from Bannister et al., 1975.

creates a hospitable environment for parasite replication (Figure 7A). Rhoptry bulb proteins (ROPs) show no resemblance between different apicomplexan species, indicating they might have evolved to suit their target host cell (reviewed by Boothroyd and Dubremetz, 2008).

Finally, the dense granules secrete their protein content (Carruthers and Sibley, 1997) and provide further molecules for PV formation and the remodelling of the host cell, to provide a hospitable environment for the parasite (Carruthers, 1999; Soldati et al., 2004).

1.3.2.3 Host cell egress

After the parasite has replicated and matured within its host cell, the new merozoites start to egress from the red blood cell. The outburst from a red blood cell is a two-step process: (i) the parasite has to disrupt the parasitophorous vacuole and then (ii) break apart the host cell membrane (Wickham et al., 2003). The host cell cytoskeleton is destabilised and the membrane eventually disrupted due to an increase in intracellular pressure and biochemical changes (Glushakova et al., 2005). The exact mechanisms behind these events still remain largely unknown. One of the protein families, that are likely to be involved in the biochemical pathways leading to parasite egress, are SERA family proteases (Salmon et al., 2001). Members of this family localise to the PV membrane and are known to be involved in sporozoite egress from oocysts (Hodder et al., 2003; Miller et al., 2002). Proteolysis of SERA proteins themselves is controlled by the protease PfSUB1, a protein secreted by the exonemes during activation of host cell egress (Koussis et al., 2009; De Monerri et al., 2011; Yeoh et al., 2007). These apical organelles have only recently been discovered in merozoites (Singh et al., 2007) and are likely to be present in sporozoites as well, due to their similar invasion processes. Eventually, when the host cell cytoskeleton is completely destabilised an explosive disruption of the host cell disperses the nonmotile

merozoites into the blood stream ready to invade another erythrocyte. During host cell egress the parasite is already preparing for the next round of invasion. For example, PfSUB1 is also involved in the primary proteolytic processing of MSP-1 (Merozoite surface protein 1), a protein that is involved in securing irreversible host cell attachment (see below) (Child et al., 2010). These processes have been shown to be essential for parasite invasion, their exact function, however remains unknown.

1.4 Armadillo repeat proteins

Armadillo (ARM) repeat proteins form a diverse family of proteins that are involved in many different cellular mechanisms, performing several independent roles. These proteins are characterised by a tandem repeat of 42 amino acids, forming the ARM repeat domain. These tandem repeats create a conserved three dimensional structure: one single ARM repeat forms three α-helices, the α-helices of several tandem repeats then build a right-handed superhelix (Figure 8B) (Peifer et al., 1994). This structural element within the proteins acts as a versatile platform for different protein-protein interaction, enabling the proteins to perform their diverse roles.

Despite the highly conserved tertiary structure, ARM repeat proteins do not necessarily share the same sequence identity (Figure 8A) (Kidd et al., 2005; Striegl et al., 2010), which is why members of this protein family are difficult to identify using bioinformatics methods. Through crystal structure analysis, however, several representatives of this protein family were identified (Choi and Weis, 2005; Conti et al., 1998; Huber et al., 1997). Some of those are discussed in the following sections in more detail.

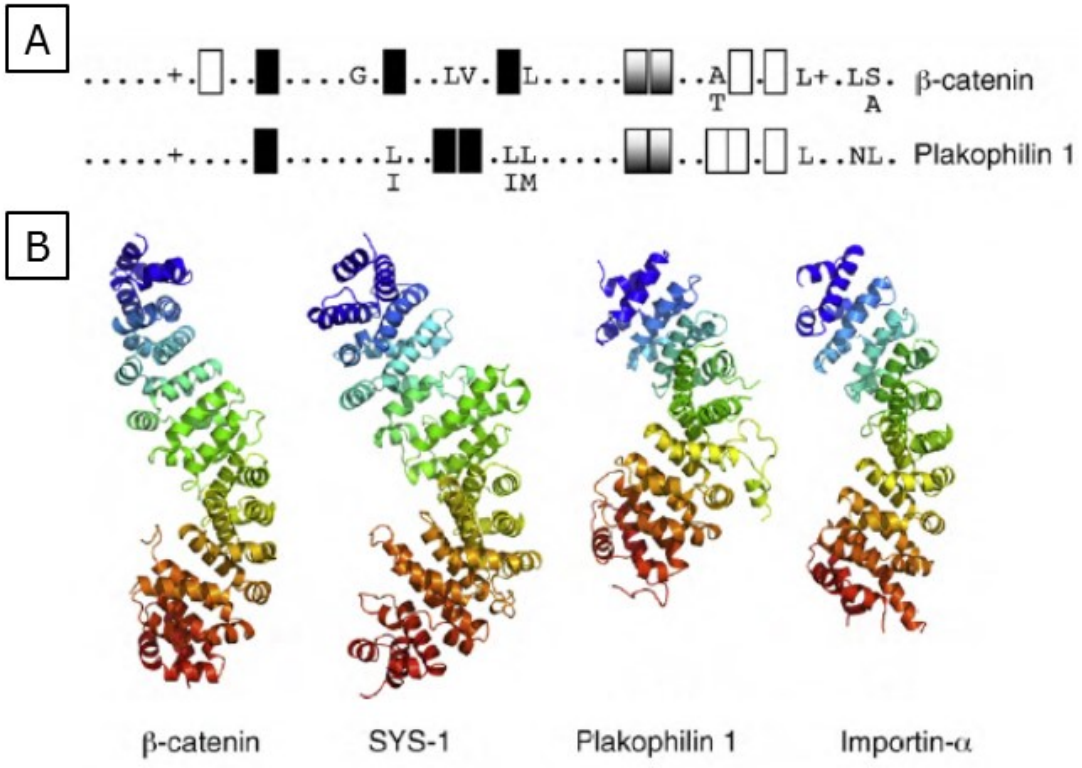


Figure 8| Structure of Armadillo repeat proteins: ARM repeat proteins only share a low amount of sequence similarity. However, some positions in the primary protein sequence are reserved for amino acids with specific properties as indicated in panel **(A)**. (+ → basic (H, K, R); white square → small hydrophobic (A, C, P, V, T); black square → large hydrophobic (F, I, L, M, W); two-tone square → any hydrophobic) Despite the lack of sequence similarity all ARM repeat proteins share a common 3D-structure. One ARM repeat forms three α-helices, which then assemble to form a superhelix structure. **(B)** Image adapted from Tewari et al., 2010.

1.4.1 Armadillo repeat proteins across the animal kingdom

1.4.1.1 β-catenin

β-catenin is the classical prototype for an ARM repeat protein. It is conserved across the whole animal kingdom and is involved in different cellular mechanisms through its protein-protein interactions. Its ARM repeat domain is located in the centre of the protein and contains 12 ARM repeats that are highly conserved across species. The ARM domain is positively charged and forms a groove that provides the typical platform for binding partners (Graham et al., 2000). Its negatively charged N-terminal domain provides a binding site for α-

catenin and several phosphorylation sites (Jiang and Struhl, 1998; Wu et al., 2003). The N-terminal domain of β-catenin is very flexible and can therefore act as a regulator of interactions between the ARM domain and possible binding partners (Huber et al., 1997). Because of its flexibility and opposite charge, the N-terminal domain can bind to the ARM domain, inhibiting the interaction with any other proteins and therefore regulate the function of β-catenin.

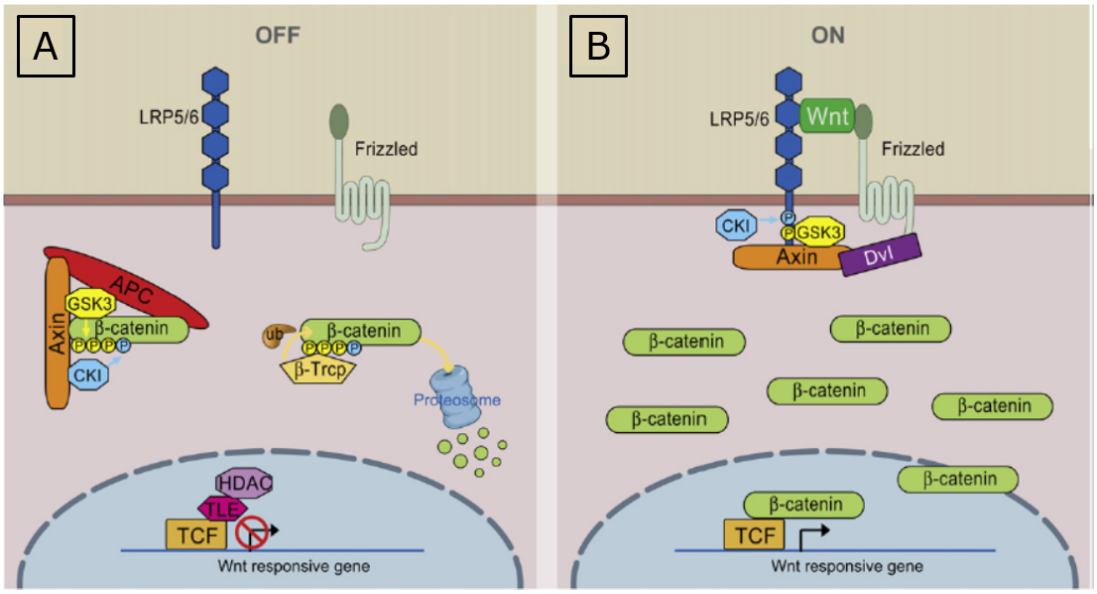


Figure 9| β-catenin signalling: β-catenin is an ARM repeat protein that acts as a coactivator for TCFs of Wnt-target genes. In the absence of a Wnt signal **(A)** β-catenin is phosphorylated by a complex of axin, APC, CK1 and GSK3 which eventually leads to its proteosomal degradation and the repression of Wnt target genes. If the cell, on the other hand, receives a Wnt signal **(B)**, the phosphorylating complex gets bound by the LRP5/6-Frizzled receptor complex and β-catenin is free to regulate the gene expression in the cell nucleus. Image adapted from MacDonald et al., 2009.

β-catenin acts as a regulator of cell-cell adhesion. With its ARM repeat domain, it is able to bind the cytoplasmic domain of cadherin (calcium-dependent adhesion protein) and is thereby indirectly associated with adherens junctions (protein complexes in cell-cell junctions of epithelial and endothelial tissues). β-catenin also regulates the homodimerization of α-catenin, which allows it to take control of actin branching and bundling, an essential part of cell-cell adhesion (Korswagen et al., 2000; Natarajan et al., 2001).

Secondly β-catenin is involved in the regulation of gene expression through the Wnt-signalling pathway (Figure 9). Briefly (reviewed by MacDonald et al., 2009), in the absence of the Wnt-protein ligand cytoplasmic β-catenin forms a complex with axin (tumour suppressor gene), APC (*adenomatous polyposis coli* gene product), CK1 (casein kinase 1) and GSK3 (glycogen synthase kinase 3), which leads to a phosphorylation of β-catenin through CK1 and GSK3. Phosphorylated β-catenin is recognised by the E3 ubiquitin ligase β-Trcp, which leads to a proteosomal degradation of the ARM repeat protein. Without β-catenin in the nucleus the expression of Wnt target genes is repressed by TCF-TLE/Groucho and HDAC (transcription factors) (Figure 9A). If, however, a Wnt signal reaches the cell, the ligand binds to the F_z-LRP5/6 receptor complex. This activation leads to the recruitment of Dvl (cytoplasmic phosphoprotein) and the phosphorylation of the LRP5/6 receptor causing the recruitment of axin. With axin bound to the receptor complex, β-catenin no longer interacts with the dephosphorylating complex and is free to accumulate in the cell nucleus, where it acts as a coactivator of TCFs driving the expression of Wnt target genes (Figure 9B).

The Wnt-signalling pathway is essential for embryonic development and stem cell maintenance and is therefore associated with multiple diseases in case of its dysregulation. This example perfectly highlights the essential and diverse roles ARM repeat proteins play within a cell.

1.4.1.2 Importin-α

Importin-α is another well studied protein that contains ARM repeats. There are three phenotypically distinct isoforms of Importin-α (α1, α2, α3). All of these groups can be found in humans, however only Importin-α1 is conserved in all eukaryotes, while Importin-α2 and Importin-α3 can only be found in Metazoans

(Malik et al., 1997; Mason et al., 2002), suggesting that these isoforms have evolved during the development of multicellular organisms.

Importin-α contains ten ARM repeats, that form the typical groove for protein-protein interactions. An IBB (Importin-β binding) domain is located at the N-terminal end of the protein and is essential for forming the functional complex with Importin-β (Conti et al., 1998; Fontes et al., 2000; Herold et al., 1998). The Importin-α/β-complex plays a pivotal role in the transportation of cargo-proteins into the nucleus. During this process, the ARM repeat domain in Importin-α acts as the NLS (nuclear localization signal) binding site, recognising and attaching the cargo protein (Weis et al., 1996).

Similar to β-catenin this function can be regulated by the protein itself (Kobe, 1999). The N-terminal IBB domain is highly flexible and can bind to the ARM domain and thereby blocks the protein's ability to bind NLS-proteins.

1.4.1.3 ARM repeat proteins and the cytoskeleton

As mentioned above ARM repeat proteins are involved in different cellular processes due to their versatile interaction platform. The following sections provide an overview of known ARM repeat proteins across different species involved in particular cellular events and mechanisms.

The afore mentioned β-catenin has already shown the essential functions of ARM repeat proteins within the cytoskeleton. However, it is not the only identified ARM protein known to be involved in the maintenance of cell stability and cell movement. Vac8p is a yeast-protein that shares a 22% sequence identity with β-catenin and therefore is likely to play a similar role (Wang et al., 1998). Research has shown that Vac8p acts as a link between intracellular membranes and the actin cytoskeleton and is therefore directly involved in the maintenance of cell stability and organisation. It can also be found at inter-organelle junctions (e.g. nuclear membrane and vacuole) where it binds to the integral membrane

protein Nvjp and is responsible for its localisation (Pan et al., 2000). In other Protozoa the protein Nd9p can be found, which is closely related to Vac8p, regulating the exocytic membrane fusion between trichocysts and plasma membranes (Froissard et al., 2001).

PF16 is a protein containing nine ARM repeats. It has been shown to be required for cellular motility, by localising to the C1 microtubule in the flagella of Protozoa (Smith and Lefebvre, 2000). It is one of the few ARM repeat proteins that is conserved and well-studied in apicomplexan parasites and will therefore be further discussed in chapter 1.4.2.1. The homologue of PF16 in mammals, SPAG6, is a testis-specific sperm protein. Similar to PF16, it interacts with the central pair of microtubules in the sperm-tail and SPAG6 depleted mice have been shown to be infertile, highlighting the importance of the protein in sperm motility (Sapiro et al., 2000).

The vertebrate family of p120 proteins contains ARM repeat proteins that are similar to β-catenin in structure and function (Anastasiadis and Reynolds, 2000). Like β-catenin they bind cadherins using their ARM repeat domains and stabilising the surface cadherin cluster. With this interaction the endocytosis and degradation of cadherins is blocked. This gives p120 proteins the ability to directly regulate cell-cell-adhesion. Additionally, the p120 catenin is able to affect the Rho family GTPases (Anastasiadis and Reynolds, 2001) and thereby regulate the actin cytoskeleton and the balance between cell motility and adhesion.

1.4.1.4 ARM repeat proteins and gene expression

Similar to β-catenin p120 also plays a role in gene expression, however its role and mechanisms are much less understood. Distinct isoforms of p120 have been detected in the nucleus and this localisation is regulated by alternative splicing. Through the controlled integration or splicing of exons, isoforms with or without an NLS sequence are translated and either localise to the nucleus or the

cytosol, depending on their structural domains (van Hengel et al., 1999). P120 is thought to interact with the transcription factor POZ and thereby inhibit the bond between Kaiso, a DNA methylation-dependent transcriptional repressor and the DNA itself (Daniel and Reynolds, 1999; Daniel et al., 2002). The exact mechanism leading this interaction, however, remains mostly unknown.

Other known ARM proteins that have a role in the regulation of gene expression are At5g13060 in *Arabidopsis*, a protein with a similar transcriptional role as the p120-Kaiso complex (Coates, 2003), and KAP3 in Protozoa, a protein involved in chromosome segregation during mitosis through MAP-kinase signalling.

1.4.1.5 ARM repeat proteins and proteomics

Next to cell stability, motility and gene expression ARM repeat proteins have also been shown to be involved in protein degradation. The family of ARM HECT proteins consists of ubiquitin ligases, present in eukaryotes. In Arabidopsis the KAK-TUS/UPL3 complex is involved in DNA replication and trichrome branching (Coates, 2008). UFD4 (Ubiquitin-Fusion-Degradation 4), an ubiquitin ligase with similar function can be found in yeast (Ju and Xie, 2006). In Protozoa and mammals ARMC8, an 8 ARM repeat containing protein, is part of the CTLH complex and involved in proteasome/polyubiquitin-dependent protein degradation (Kobayashi et al., 2007; Suzuki et al., 2008). The ARM repeats initiate an interaction between the membrane/receptor proteins and the signalling adapter, leading to mono-ubiquitination and degradation.

1.4.2 Armadillo repeat proteins in Apicomplexa

As the studies of the protein family in different eukaryotic cells have shown, ARM repeat proteins play essential roles in different cellular events and processes. Despite their known importance, however, they have hardly been investigated in Apicomplexan parasites. In 2010, Tewari et al., have identified ten ARM repeat proteins in Apicomplexan parasites, five of these are specific for the phylum (Figure 10). The specific proteins are interesting in particular, because these might act as potential drug targets. In this sections characterised ARM repeat proteins in apicomplexan parasites are discussed in more detail and the central protein of interest of this thesis is introduced.

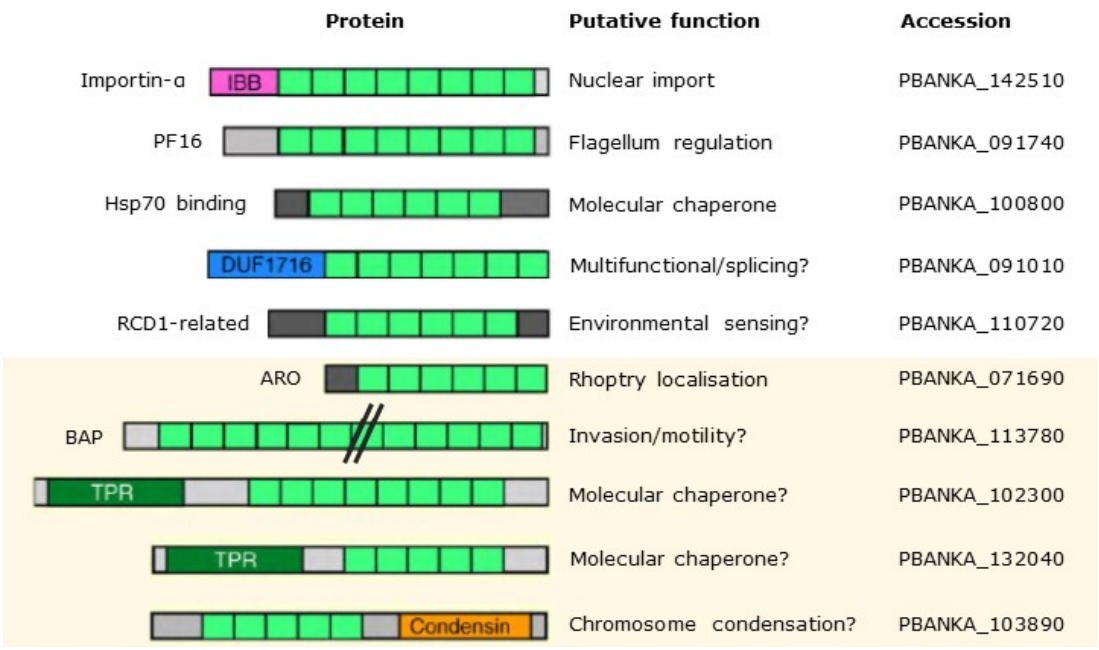


Figure 10| Armadillo repeat proteins in Apicomplexan parasites: Ten putative ARM repeat proteins were identified in Apicomplexan parasites using bioinformatics analysis. Five of these proteins are specific for the phylum of Apicomplexa (marked with orange background). Indicated are the domain structure, putative function and the accession number for each *Plasmodium berghei* protein in PlasmoDB. Abbreviations: IBB → importin-β binding; DUF1716 → domain of unknown function 1716; TPR → tetratricopeptide repeats. Image adapted from Tewari et al., 2010 and modified.

1.4.2.1 PF16



PF16 is an ARM repeat protein that is conserved throughout different species. As described in chapter 1.4.1.3 its mammalian homologue has been shown to play an essential role in sperm motility of male mice. In 2010, Straschil et al., have shown a similar role of PF16 in the flagella of male *Plasmodium* gametes. The flagellum is an ancient cellular organelle, necessary for cell motility. It consists of a central apparatus, a pair of microtubules C1 and C2 (the axoneme), that is encircled by nine doublet microtubules with Dynein arms that regulate motility. The flagella of *Plasmodium* gametes are assembled in the cytosol and attached to the membrane during exflagellation. Eventually the long, thin and motile gametes almost entirely consist of flagella.

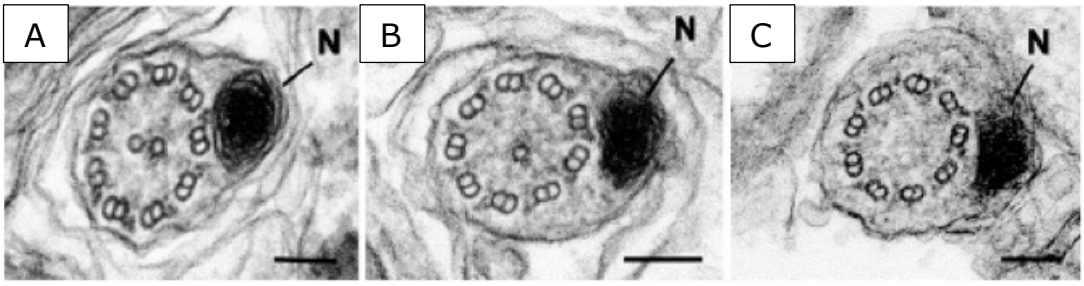


Figure 11| PF16 is essential for flagella composition and function: A knockout of PF16 has a severe impact on flagella structure of *Plasmodium*. In a WT parasite the axoneme contains two central microtubules C1 and C2 **(A)**. Upon the depletion of PF16 the flagella lose one **(B)** or both **(C)** of their central microtubules and are impacted in motility and fertilization of female gametes. Image was adapted from Straschil et al., 2010.

The protein was first functionally characterised in *Chlamydomonas*, where its inactivation caused the loss of the central pair of microtubules in the flagella. This caused abnormal function and movement of the flagella, indicating an essential role of the protein in flagellar motility and stability of the axoneme (Smith and Lefebvre, 1996). In *Plasmodium* 50% of PF16 depleted male gametes were nearly immotile, showing a lower beat frequency and amplitude, as well as a reduced speed. However, this did not result in a total block of fertility, suggesting

that PF16 is needed for motility of the gametes but not for the process of fertilization itself. PF16KO parasites showed a similar structural phenotype as Chlamydomonas: over 60% of gametes lost both of their central microtubules; another 20% were depleted of one. Only 12% of parasites did not show an obvious phenotype (Figure 11). This phenotype corresponds with the function of PF16 seen in other species and highlights the importance of this conserved ARM repeat protein in *Plasmodium* parasites.

1.4.2.2 Armadillo repeat only (ARO)



In 2014 Mueller et al., have characterised an Apicomplexan specific ARM repeat protein in *Toxoplasma gondii* and named it ARO (Armadillo repeats only protein). The protein consists of two predicted ARM repeats and is highly conserved throughout apicomplexan parasites.

Their research demonstrated a localisation of ARO to the rhoptries in *T. gondii.* The acylation motifs of ARO have been shown to be essential for this localisation, indicating a membrane attachment through the covalent attachment of fatty acids. ARO is located to the bulb and the neck of the rhoptry and is only present at mature organelles.

Early experiments in the study have proven the protein to be essential for the parasite's survival, but not necessary for the parasite's growth. However, ARO depleted parasites were significantly impaired in host cell invasion and attachment. These defects, as shown by further research, were caused by a mislocalization of the rhoptries within the parasite. Instead of being attached to the apical end of the parasite the organelles were dispersed throughout the cytosol. As described in section 1.3.2 the rhoptry organelles contain proteins, essential for the process of invasion. Without a proper apical attachment, the organelles are no longer able to secrete their content into the host cell and the

parasite cannot form a stable connection to the host cell. Therefore, the invasion process cannot be initiated. Mutation of the glycine residues within the acylation motif of ARO, which are necessary for myristoylation and palmitoylation, caused the same phenotype, indicating that the attachment of ARO to the rhoptry membrane is essential for the organelle's localization.

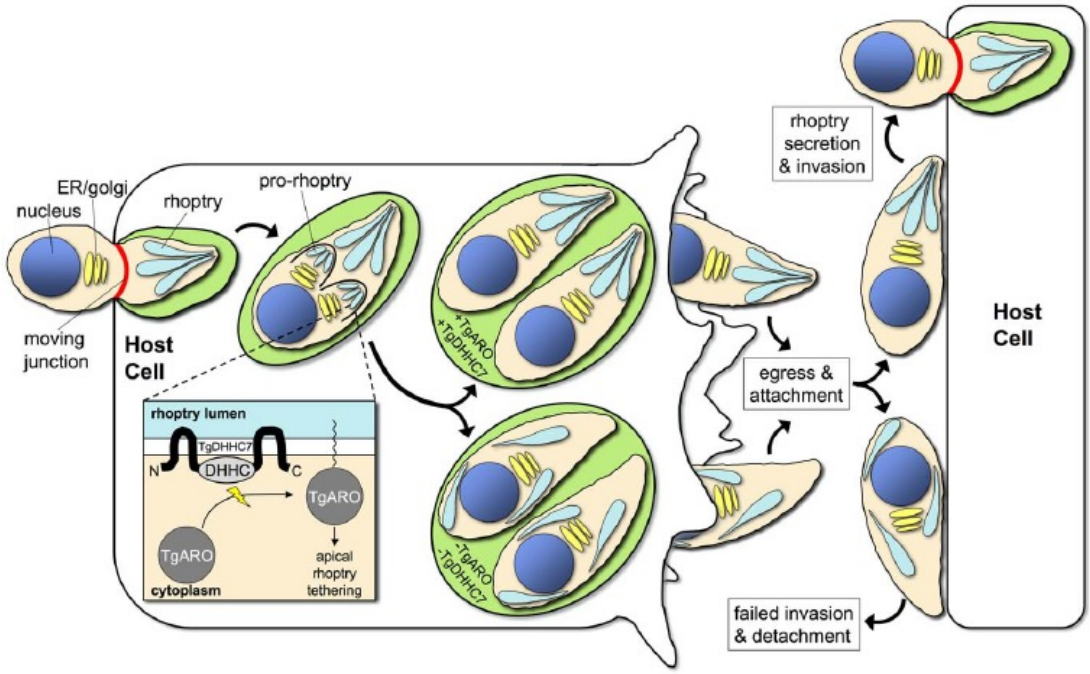
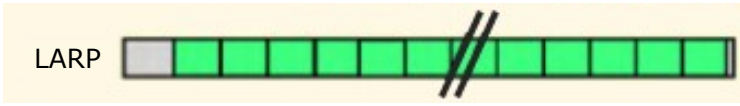


Figure 12| Role of ARO in *Toxoplasma gondii*: Beck et al. hypothesize that TgARO is recruited to the rhoptry membrane through palmitoylation by TgDHHC7, where it secures the attachment of the rhoptry organelles to the apical end of the parasite. Upon ARO or TgDHHC7 depletion the rhoptries are dispersed in the cytosol and the parasite is no longer able to invade host cells. Image adapted from Beck et al., 2013.

After the identification of different ARO interaction partners Beck et al., proposed a model where ARO is recruited to the rhoptry surface by the Palmitoyl acyl transferase TgDhhC7 through palmitoylation and dock the rhoptries to the apical end of the parasites (Figure 12). There were other ARO interaction partners identified as well (e.g. Myosin F, TgACβ (adenylyl cyclase)), TgDhhC7 however remains the most well studied interaction.

These recent studies have highlighted the variety and importance of different ARM repeat proteins in Apicomplexan parasites and have proven that this protein family is worth further investigation to understand apicomplexan biology.

1.4.2.3 PBANKA_113780



PBANKA_113780 is a hardly characterised, apicomplexan-specific ARM repeat protein with unknown function. With a molecular weight of 291 kDa it is the biggest, identified ARM repeat protein in Apicomplexa and contains the largest number of ARM repeats. Therefore, we have named this protein **L**arge **A**rmadillo **r**epeat **p**rotein (LARP) to simplify future reference in this thesis.

This protein is the central focus of this Master of Research Project to further identify the functional properties and roles of ARM repeat proteins in apicomplexan parasites. The results to this study are presented in the following chapters.

2 Aims and Objectives

Until today, the biology and development of the malaria parasite *Plasmodium* is not completely understood. For future treatment and prevention of malaria infections, the cell biology and biochemistry of *Plasmodium* have to be further investigated. A significant number of *Plasmodium* genes have never been characterised and their function is completely unknown. Therefore, the main focus of this lab's research is the analysis of known and unknown regulatory genes and proteins and the identification of their function through cell biological, biochemical, proteomic and reverse genetic approaches.

This project focuses on the family of ARM repeat proteins as their importance and diversity is already well known and understood in other eukaryotes (Coates, 2003; Tewari et al., 2010). In recent years, first studies on ARM repeat proteins (e.g. PF16 and ARO) in Apicomplexa have highlighted the pivotal role this protein family could play in these unicellular parasites. Earlier research in the lab has identified ten ARM repeat proteins in Apicomplexan parasites, five of which are specific to the phylum (Tewari et al., 2010). The **l**arge **A**rmadillo **r**epeat **p**rotein (LARP) is the largest ARM repeat protein known in Apicomplexa and contains approximately 60 ARM repeats (Tewari et al., 2010). ARM repeat domains are known to act as a versatile platform for protein-protein interaction and this high number of repeats could enable the protein to play a key role in important interactions and cellular mechanisms. Additionally, genome wide RNAseq studies (Otto et al., 2014) have identified LARP as one of the highest expressed genes in *Plasmodium* schizont and other asexual blood stages, the life cycle stages that are causing the clinical symptoms of malaria. Therefore, the main aim of this thesis is to characterise the cell biology of LARP in the model system *Plasmodium berghei*.

This aim is approached through three different objectives. Firstly, the localisation of LARP in the *Plasmodium berghei* parasite will be analysed throughout its complete life cycle, by endogenously tagging the C-terminus of the protein with GFP. For more detailed information, this study will be expanded by using known cellular markers and proteins as a reference for the cellular localisation. Secondly, biochemical properties of LARP will be investigated. Through the use of subcellular fractionation, the exact subcellular localisation and solubility can be determined, which could hold important information on possible modifications of the protein. Finally, a functional analysis will be performed to propose a role for LARP within the *Plasmodium berghei* parasite. Through a complete gene knockout or a conditional knockdown approach the defects caused by a gene depletion can be assessed. Based on these results, a connection between LARP and specific mechanisms and processes in the *Plasmodium* parasite could be drawn.

3 Material and Methods

Table 1| List of chemicals

Chemical	Manufacturer
Agarose	Invitrogen
β-mercaptoethanol	Sigma-Aldrich
Bradford reagent	Sigma-Aldrich
Bovine serum albumin	Melford
CF11	Whatmann
DEPC treated water	Invitrogen
dNTPs	Invitrogen
EDTA free protease inhibitor	Roche Diagnostics
Ethanol	Sigma-Aldrich
Ethidium bromide	Fisher Scientific
Fetal Bovine Serum (FBS)	Sigma-Aldrich
Giemsa Stain	Sigma-Aldrich
Heparin	Sigma-Aldrich
Hoechst	Sigma-Aldrich
Isopropanol	Fisher Scientific
Methanol	Sigma-Aldrich
NP-40	MP Biomedicals
NycoDenz	Axis-Shield PoC
Paraformaldehyde	Electron Microscopy Science
Penicillin/Streptomycin	Sigma-Aldrich
Phenol/chloroform	Sigma-Aldrich
Phenylhydrazine	Sigma-Aldrich
Poly-L-lysine	Sigma-Aldrich
Pyrimethamine	Sigma-Aldrich
RPMI 1640	Life Science Technologies
Sodium acetate	Fisher Scientific
Sulphadiazine	Sigma-Aldrich
Triton X-100	Sigma-Aldrich

Table 2| List of solutions

Solution	Composition
Carbonate solution	0.1 N Na_2CO_3 pH 11.0, complete protease inhibitor cocktail
CLB	20 mM Hepes, 20 mM Glucose, 4 mM sodium bicarbonate, 1 mM EDTA, 0.1% BSA, pH 7.35 in PBS
Electrophoresis buffer	15.1 g Trisbase, 94 g glycine, 25 ml 20% SDS per litre
Freezing solution	9 parts Alseverver's solution plus 1 part glycerol
Hypotonic solution	10 mM TRIS, pH 8.0, 5 mM EDTA, complete protease inhibitor cocktail
Laemmli buffer	Tris/HCl pH 6.8, Glycerol, 2% SDS, 2-mercaptoethanol, bromophenol blue
LB medium	10 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl (in 950 mL H_2O)
Microtubule-stabilising buffer	10 mM MES pH 7, 150 mM NaCl, 5 mM EGTA pH 7, 5 mM MgCl2, 5 mM glucose
Nycodenz	5.0 mM Tris, 3.0 mM KCl, 27.6 % w/v Nycodenz, pH 7.2
Ookinete culture medium	4 µg/mL sodium bicarbonate, 0.1 mM xanthurenic acid, 3 U/ mL penicillin/streptomycin, pH 7.4 in RPMI
Red blood cell (RBC) lysis buffer	150 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 1 mM EDTA pH 8
Schizont culture medium	25% foetal calf serum, 3 U/mL Penicillin/Streptomycin pH 7.3 in RPMI
SOC medium	20 mM glucose, 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl (in 950 mL H_2O)
Solution I	50 mM glucose, 25 mM Tris-HCl pH8, 10 mM EDTA pH8, 100 µg/ml RNase A
Solution II	0.2 M NaOH, 1% SDS
Solution III	3 M potassium acetate, 11.5 % acetic acid
Tris-acetate-EDTA (TAE) buffer	4.84 g/L Tris, 1.142 mL/L acetic acid, 1 µM EDTA pH 8
Tris-borate-EDTA (TBE) buffer	10.8 g/L Tris, 5.5 g/L boric acid, 2 µM EDTA pH 8

Tris-EDTA (TE) buffer	10 mM Tris, 1 mM EDTA pH 8
Tris-NaCl-EDTA (TNE) buffer	10 mM Tris, 100 mM NaCl, 5 mM EDTA pH 7.5
Transfer buffer	3 g/L Tris, 14.4 g/L glycine, 20% Methanol

Table 3| List of commercial kits

Kit	Manufacturer
Fast SYBR® Green Master Mix	Applied Biosystems™
GenElute™ Plasmid Miniprep Kit	Sigma-Aldrich
GFP-Trap®_A	Chromotek
High-Capacity RNA-to-cDNA™ Kit	Applied Biosystems™
Nucleofector™ Kit for Human T Cells	Lonza
QIAquick® PCR purification kit	QIAGEN
RNase-Free DNase Set (50)	QIAGEN
RNeasy® Mini Kit	QIAGEN
SilverQuest™ Silver Staining Kit	Novex™
Western Breeze Chemiluminescent Anti-Rabbit Kit	BioRad

Table 4| List of enzymes

Enzyme	Buffer	Manufacturer
ApaI	A or Multicore	Promega
BglII	C	Promega
EcoRI	CutSmart	NEB
EcoRV	D or Multicore	Promega
HindIII-HF	CutSmart	NEB
KpnI	1.1 or CutSmart	NEB
NaeI	CutSmart	NEB
NheI	2.1 or CutSmart	NEB
NotI-HF	CutSmart	NEB
Phusion	HF buffer	NEB
Proteinase K	-	Sigma-Aldrich
PstI-HF	CutSmart	NEB
RNase A	-	Sigma-Aldrich
SacII	C	Promega

T4 DNA ligase	T4 DNA ligase buffer	NEB
XbaI	D, E or Multicore	Promega
XhoI	CutSmart	NEB

Table 5| List of primers

Primer sequences are given in 5′ – 3′ direction. Lowercase letters indicate restriction sites.

Primer name	Sequence	Experiment
3′IntAMA1	TGTGTATATATAAGTATTGTATGGTTG	LARP-PTD integration PCR
5′IntPTD	TCTACTTTATTTGCTAATTCTGG	LARP-PTD integration PCR
Int3′PTD011	ATTTTCATCATTTAAAGCCTTCGAACCA	LARP-PTD and LARP-Tet integration PCR
Int5′PTD011	TTTCGATTTATTCCCATATGCGCAATA	LARP-PTD integration PCR
Int5′PTD011B	GTATAACACGTTTTTTTTATGAAATATCT	LARP-Tet integration PCR
IntPROM	AATAGAAATATATCAGGATCGCCAG	LARP-Tet integration PCR
IntT135	AATGGTGGGTCTGTTTTGCT	LARP-GFP integration PCR
IntTRAD	TTATTCATGTCGTTGTAGTACGGG	LARP-Tet integration PCR
N041-1	CCCCGGGCCCGTACCATATATCGATGATACAGATGCC	Cloning of KO construct
N041-2	GGGGAAGCTTCAACTTCGCAGTATGTGTATCTGATGTG	Cloning of KO construct
N041-3	CCCCGAATTCCCTTCATATCGAAAACGTCCATATGTATAT	Cloning of KO construct
N041-4	GGGGTCTAGACTAAAAGTGCCTTTTCAGCGTGCCTC	Cloning of KO construct
ol248	GATGTGTTATGTGATTAATTCATACAC	LARP-PTD integration PCR
ol492	ACGCTGAACTTGTGGCCG	LARP-GFP integration PCR
PTD011-1	gagaccgcggTACATTATTACACTTGGTGTTTTAGTTAC	Cloning of PTD construct
PTD011-2	gagactgcagTTCCTTAGATATAATATAATAACAAACAGTATG	Cloning of PTD construct
T135-1	ccccggtaccGAATCATTTCCAGATGCAGAATTAGAAACAGG	Cloning of GFP tagged construct

T135-2	ccccgggcccATAGTTATAACTAGCA GCATCACGCCAAAGAATTAATAGA TCGG	Cloning of GFP tagged construct
Tet001-1	gagaagatctCACCTTAAGCAAATAT TAGTTTGGAA	Cloning of Tet construct
Tet001-2	gagaccgcggTTCCTTAGATATAATA TAATAACAAACAGTATG	Cloning of Tet construct
Tet001-3	gagagccggcATGCCAAGAATTCAA GGTAAAAATTACAA	Cloning of Tet construct
Tet001-4	gagagctagcTGATGTGAACACATTT AATAAAGCAA	Cloning of Tet construct

Table 6| List of antibodies

Name	Host	Dilution	Manufacturer
13.1 (anti-P28)	mouse	IFA: 1:1000	(Tewari et al., 2005)
anti-actin	mouse	IFA: 1:500	Dr. Gerisch Laboratory[1]
anti-GFP	rabbit	WB: 1:1250 IFA: 1:250	Life Technologies
anti-GFP	mouse	WB: 1:1250 IFA: 1:250	Roche Diagnostics
anti-H4K20Me3	rabbit	IFA: 1:500	Abcam
anti-H4K20Me3	mouse	IFA: 1:500	Abcam
anti-mcherry	mouse	WB: 1:1250	Chromotek

Table 7| List of materials for microscopy

Material	Source
Coverslips	Zeiss
Fluoromount-G	SouthernBiotech
Poly-L-lysine coated microscopy slides	Sigma-Aldrich

[1] Max Planck Institute of Biochemistry, Martinsried (Delbac et al., 2001)

3.1 Ethics statement

All animal work at the University of Nottingham has passed an ethical review process and was approved by the United Kingdom Home Office. Work was carried out in accordance with the United Kingdom 'Animals (Scientific Procedures) Act 1986' and in compliance with 'European Directive 86/609/EEC' for the protection of animals used for experimental purposes under UK Home Office Project Licenses 40/3344 and 30/3248.

3.2 *Plasmodium berghei* strain

All work was performed using the *Plasmodium berghei* ANKA strain. *P. berghei* parasites were originally isolated from thicket rats in Central Africa. Transgenic parasites were generated in strain 2.34 parasites, originally provided by Prof. Robert Sinden (Imperial college). Parasites were either contained in NIHS or MF1 outbred mice and transmitted by *Anopheles stephensi*.

For generation of transgenic parasites *P. berghei* ANKA *wild-type* (WT) strain or *wild-type-gfp* (WT-GFP) were used. The latter (originally obtained from Dr. Chris Janse, University of Leiden) expressed GFP in every life cycle stage under the control of the promoter of the *elongation factor 1 alpha (eef1a)* gene.

3.3 Mouse maintenance

Six-to-eight week old female NIHS or MF1 outbred mice were used for all experiments. Mice were kept in a 12h light-dark cycle in ventilated cages at 21-23°C. After infection with *Plasmodium berghei* parasites parasitaemia (percentage of red blood cells infected by parasites) was monitored daily via tail blood smears stained in Giemsa. Briefly, tail blood smears were fixed in methanol for 5 min,

incubated in Giemsa stain (~1:10) for 10 min and assessed with a Nikon Phase Contrast microscope.

3.4 Mosquito maintenance

Anopheles stephensi mosquitoes (strain SD500) were bred and grown in an environment of 26-28°C with a humidity of 30-40%. Larvae were grown in plastic trays containing salt water and transferred into a netted cage as soon as they reached the pupae stage. In netted cages pupae were allowed to grow into mosquitoes. Larvae were fed daily with fish food, mosquitoes received a constant source of 2% glucose.

3.5 Generation of transgenic parasites

3.5.1 Polymerase chain reaction (PCR) for DNA amplification

To generate constructs for the genetic manipulation of *Plasmodium berghei* fragments of the parasite genome were amplified and inserted into a vector using a restriction digest (see chapter 3.5.2).

Wildtype (WT) DNA regions were amplified in a 50 µL PCR reaction containing 10 µL of Phusion® buffer, 2 µL of dNTP's (200 µM), 2.5 µL of the forward and the reverse primer (10 pmol/µL), 1 µL of Phusion® and 2 µL of WT DNA (~50 ng/µL). Fragments were amplified with 30 PCR cycles of 45 sec denaturation at 95°C, 45 sec annealing at 52°C and 45 sec elongation at 62°C. For the GFP-tagging construct a 1,560 bp fragment directly upstream of the TAA-stop codon was amplified using primers T135-1 and T135-2 containing an *ApaI* and *KpnI* restriction site (see Table 5). For the knockout construct a 532 bp fragment

containing an *ApaI* and a *HindIII* restriction site was amplified directly upstream of the ATG-start codon using primers N041-1 and N041-2. A second fragment of 478 bp was amplified 53 bp downstream of the TAA-stop codon using primers N041-3 and N041-4 containing an *EcoRI* and an *XbaI* restriction site. For LARP-PTD a 532 bp region directly upstream of the start codon was amplified using the primers PTD011-1 and PTD011-2 containing the restriction sites *SacII* and *PstI*. The 5' region of the LARP gene was amplified using the primers PTD011-3 and PTD011-4 containing restriction sites for *XhoI* and *NotI*, resulting in a 780 bp fragment that includes the ATG-start codon. To generate the construct for LARP-Tet a 756 bp fragment directly upstream of the start codon was amplified using the primers Tet001-1 and Tet001-2 containing the restriction sites for *BglII* and *SacII*. A second 780 bp long fragment starting at the ATG-start codon was amplified using the primers Tet001-3 and Tet001-4 with the restriction sites for *NaeI* and *NheI*.

After successful amplification the DNA-fragments were purified using the QIAquick® PCR purification kit according to the manufacturer's instructions.

3.5.2 Restriction enzyme digest

After purification of the amplified fragments, vector (chapter 3.5.3) and insert were digested with the respective restriction enzymes Table 4). The double digest mixture was set up in a total reaction of 100 µL containing 50 µL of the PCR product, 2 µL of each restriction enzyme, 10 µL of the appropriate buffer (Table 4) and 36 µL of sterile distilled water. Enzymes were used as indicated in Chapter 3.5.1. The mixture was incubated at 37°C for 1 h and afterwards purified using phenol/chloroform extraction. Briefly, 25 µL of 3M sodium acetate and 100 µL of phenol/chloroform were added to digest mixture, vortexed and spun for 1 min at full speed. The upper layer, containing DNA, was transferred to a new tube and again mixed with 25 µL of sodium acetate. For DNA precipitation ice cold 100%

ethanol was added to the solution and incubated for 20 min on ice. DNA was washed with 70% ethanol and resuspended in sterile distilled water.

The successful amplification and digest of the DNA fragment was controlled via gel-electrophoresis. A 1% agarose gel in TAE buffer containing ethidium bromide (1:50,000) for DNA detection was used and samples were ran for 35 min at constant 120 V. The agarose-gel was detected using an UV Transilluminator.

3.5.3 Ligation and transformation

Insert and vector were ligated with a 7:1 ratio in a 10 µL reaction mix containing 1 µL of T4 DNA ligase and 1 µL of T4 DNA ligase buffer. Reaction was incubated at room temperature for 2 h and a self-ligation (reaction mix without the insert) was run as a negative control.

For GFP-tagging of the gene pOB277 vector (Appendix A) was used containing a GFP-sequence immediately downstream of the restriction sites. The vector pOB90 (Appendix C), containing a human dihydrofolate reductase (*hdhfr*) cassette directly between the two UTR insertion sites, was used to generate the LARP-KO construct. For generation of the LARP-PTD the vector pSS368 (Appendix B) was used. It contains an *hdhfr* cassette directly downstream of the UTR insertion site and an *ama1* promoter sequence upstream of the gene insertion site. The LARP-Tet construct was generated using the pPRFp-TRAD4Ty-T7HAPRF-hDHFR vector (Appendix D), containing a TetO7 and HA-tag sequence upstream the gene insert site and a TRAD4 sequence downstream of the 5'UTR insertion site. All vectors contain an Ampicillin resistance gene for selection of transformed bacterial colonies and an *hdhfr* cassette providing resistance against pyrimethamine.

The ligated construct was then transformed into XL1-BLUE competent cells. Briefly, 10 µL of ligation product were mixed with 50 µL competent cells and

incubated on ice for 20 min. Heat shock was performed for 1 min at 42°C before incubation on ice for 2 min. 200 µL of SOC medium were added to the competent cells and the mixture was incubated for 1 h at 37°C while gently shaking. Cells were then spread out over a LB-agarose plate containing ampicillin for selection of colonies containing the construct and left to grow at 37°C overnight. Colonies were picked from the plates and added to LB broth containing ampicillin. Samples were left shaking overnight at 37°C and 240rpm to promote cell growth.

3.5.4 Isolation of plasmid DNA from bacteria

For confirmation of successful ligation, plasmids were isolated from bacteria cultures and tested through a digest with specific restriction enzymes. 1.5 mL of bacterial culture was spun down and the pellet resuspended in solution I. Cells were lysed by adding solution II and finally neutralized with solution III. After 15 min centrifugation at full speed the plasmid containing supernatant was transferred into a new tube, the DNA precipitated with 100% isopropanol and incubated on ice for 20 min. The pellet was washed with 70% ethanol and resuspended in sterile distilled water. Purified plasmid was then digested with two restriction enzymes (enzymes and constructs as indicated in chapter 3.5.1) in a 10 µL reaction mix containing 5 µL of DNA, 0.5 µL of each enzyme and 1 µL of the appropriate buffer (Table 4) and incubated for 3 h at 37 °C. The digest product was detected by gel-electrophoresis allowing confirmation of presence and length of the gene insert. Plasmids from positive colonies were purified again using the GenElute Plasmid Miniprep kit according to manufacturer's instructions.

3.5.5 Transfection of plasmids into parasites

For transfection of the constructs the DNA was first linearized to allow a single or double homologous recombination with the parasites DNA. Plasmids were digested at 37°C overnight in a 100 µL reaction containing 20 µL of the construct,

2 µL of each enzyme or 4 µL of enzyme if only one is used and 10 µL of the respective buffer. LARP-GFP and LARP-PTD were both digested with *EcoRV* creating a blunt end and thereby preventing mutations within the gene, LARP-KO was linearized with *ApaI* and *XbaI* and LARP-Tet was digested with *BglII* and *NheI*. DNA was purified using phenol/chloroform-extraction as described in chapter 3.5.2. 10 µL of the DNA solution were finally mixed with 90 µL of Human T cell NucleoFector™ Solution prior to transfection.

Constructs were transfected into *Plasmodium berghei* schizonts, which were isolated from mouse blood, infected with WT (for GFP and Tet construct) or WT-GFP (for KO, PTD and Tet constructs) parasites at a parasitaemia of approximately 4-5%. Blood was cultured and incubated at 37°C overnight with gentle rotation to support schizont development. Mature schizonts were isolated, as described in chapter 3.9.1, washed once in PBS and resuspended in the DNA solution. Cells were put in an electroporation cuvette and zapped with program U-033 using an Amaxa Biosystems Nucleofactor device. Electroporated cells were mixed with ~150 µL of naïve mouse blood and kept at 37°C with gentle rotation. After 30 min blood was injected into phenylhydrazine treated mice.

One day after transfection pyrimethamine (70 µg/mL) was added to the mice's drinking water. Due to the human dihydrofolate reductase (hdhfr) cassette in the transfected constructs parasites with successful integration have a resistance against the drug and can be selected from WT parasites in the mouse. Mice were monitored for 15 days and parasitaemia checked with Giemsa staining, as described in chapter 3.3.

3.6 Genotypic analysis

3.6.1 Isolation of asexual blood stages

To confirm successful integration of the construct into the parasite's genome asexual blood stages of the parasite were collected and tested with a diagnostic PCR. Mouse blood with a parasitaemia of approximately 7 – 10% was collected into heparin by cardiac puncture. The blood was then run through a CF11 column, wetted with PBS to isolate the erythrocytes from other blood components. The column was washed with PBS until no more, red blood cells could be eluted. The flow-through was spun down at 1,900 x*g* for 10 min and the pellet lysed in red blood cell (RBC) lysis buffer for 1 h on ice. After centrifugation at 1,900 x*g* for 10 min the pellet was washed in PBS and spun down at full speed for 5 min to be used for further analysis.

3.6.2 DNA isolation and diagnostic PCR

The asexual parasite pellet was resuspended in 1X TNE buffer containing 1.25% SDS and digested with RNase A (0.25 µg/µL) for 10 min at 37°C. Proteins in the sample were digested using Proteinase K (0.25 µg/µL) for 1 h at 50°C. DNA was isolated using phenol/chloroform extraction as described in chapter 3.5.2.

A PCR reaction was set up containing approximately 20 ng of isolated genomic DNA, 1 µL of each primer and 12.5 µL of GoTaq. A PCR program as described in chapter 3.5.1 was used to amplify the DNA. For the LARP-GFP DNA the primers IntT135, annealing to the genomic DNA 238 bp upstream of the inserted fragment, and ol492, binding within the inserted *gfp* sequence, were used to confirm successful integration. Two PCRs were performed for LARP-PTD to validate the integration of both 5' and 3' fragments. For the 5' integration primers Int5'PTD011, annealing to the genomic DNA 122 bp upstream of the inserted

fragment, and 5'IntPTD, binding inside the inserted *hdhfr* fragment were used. Primer Int3'PTD011, binding directly downstream of the inserted 3' fragment, and 3'IntAMA1, binding the inserted AMA1 promoter, were used to confirm 3' integration. Similar to LARP-PTD there were two reactions needed for LARP-Tet to confirm 5' and 3' integration. Confirmation of 5' integration was achieved by using Int5'PTD011B, binding 84 bp upstream of the integrated 5' UTR fragment and IntTRAD4, binding within the inserted TRAD4 sequence. For 3' integration primers IntProm, binding the minimal promoter region, and Int3'PTD011, binding just downstream the inserted gene fragment were used to confirm successful integration. Each primer sequence can be found in Table 5. For each reaction a control using WT-DNA and a control to test proper primer annealing was run at the same time. Further information on the strategy of integration PCR can be found in section 4.1.1.

3.7 Analysis of gene expression

3.7.1 RNA isolation

RNA was isolated using the RNeasy® Mini Kit from QIAGEN according to manufacturer's instructions. Briefly, the parasite pellet was lysed with RLT lysis buffer and mixed with an equal amount of 70% ethanol before transfer to a column binding nucleic acids. The column was washed with RW1 washing buffer to remove any residual cell components. DNA bound to the column was digested with DNase for 15 min at room temperature, leaving only RNA, and the column was washed twice with RPE mild washing buffer. The column was then dried and RNA finally eluted with RNase free water.

The concentration of the eluted RNA was checked on a NanoDrop and its quality assessed through gel-electrophoresis on a 1% agarose gel in TBE buffer. RNA samples were stored at -80°C until further usage.

3.7.2 Quantitative PCR (qRT-PCR)

To check mRNA expression of LARP, isolated WT-GFP RNA was first transcribed into cDNA using the High-Capacity RNA-to-cDNA™ Kit according to manufacturer's instructions. The reaction mix was incubated at 37°C for 1 h, heated up to 95°C keeping the temperature for 5 min and finally cooled down to 4°C. Resulting cDNA was diluted to a concentration of 80 ng/µL.

qRT-PCR reactions were set up in a 96 well-plate. For one reaction a master mix was prepared containing 10 µL of SYBR green master mix (Applied Biosystems™), 0.5 µL of each primer and 9 µL of DEPC treated water. This master mix was split into two wells to receive two technical replicas. For amplification of the target genes 40x cycles were performed heating the samples to first 95°C (3 sec) and then 60°C (30sec). The obtained C_T values were analysed using the $\Delta\Delta C_T$ method as described in section 4.1.2.

3.8 Proteomics and biochemical analysis

3.8.1 Protein preparation

After parasites were isolated from mouse blood, as described in chapter 3.6.1 and 3.9.1 the parasite pellet was resuspended in PBS containing complete EDTA free protease inhibitor and 1% NP-40 and incubated on ice for 20 min to lyse the cells. The protein-containing supernatant was separated from the cell pellet by centrifugation (10 min, full speed) and mixed with Laemmli buffer before being boiled at 95°C for 5 min.

3.8.2 Bradford assay

To measure protein concentration 1 µL of sample was mixed with 1 mL of ultrapure water in a plastic cuvette. One cuvette was set up with lysis or dilution buffer instead of the protein solution and used to blank the BioPhotometer. 1 mL of Bradford reagent was added to each sample and finally measured with the BioPhotometer against a standard curve.

3.8.3 Immunoprecipitation and Western Blot

A Western Blot was either performed from total protein lysate as described in chapter 3.8.1 or after immunoprecipitation using agarose beads coupled with anti-GFP V_HH.

Immunoprecipitation was performed using the GFP-Trap®_A kit according to the manufacturer's instructions. Briefly, cells were treated with lysis buffer and incubated on ice for 30 min while extensively pipetting every ten minutes. Cells were centrifuged at 20,000x*g* for 10 min at 4°C. The lysate was transferred into a new tube and mixed with dilution buffer in a 2:3 ratio. Protein concentration of the lysate was measured with a Bradford assay as described in chapter 3.8.2. GFP-Trap®_A beads were washed three times in ice cold dilution buffer (centrifugation: 2.500x*g*, 2 min, 4°C) before adding the protein lysate and incubating at 4°C while tumbling end-over-end to ensure proper binding between protein and beads. After 1 h beads were centrifuged and washed three times with dilution buffer. Beads were finally resuspended in dilution buffer mixed with Laemmli buffer and boiled for 10 min at 95°C to separate the proteins from the beads. After centrifugation the protein containing supernatant was transferred into a new tube and stored at -80°C until further usage.

Protein samples were run on a SDS-Page before transfer onto a nitrocellulose membrane. Protein samples were boiled for 5 min at 95°C to

denature any secondary or tertiary structures. A Protean Tetra-Cell system (BioRad) was loaded with 1x electrophoresis buffer. A 4-15% pre-cast SDS-polyacrylamide gel (Bio-Rad) was used to separate protein samples alongside a ColourPlus pre-stained protein ladder (NEB) ranging from 10 to 245 kDa. Gels were run at 175V for approximately 35 min until the dye reached the end of the gel. Proteins were then transferred onto a Hybond ECL nitrocellulose membrane (Amersham) using semi-dry blotting. Two sheets of thick Whatmann paper and the membrane were soaked in 1x transfer buffer and loaded onto the semi-dry blotting apparatus in the following order: Whatmann paper, membrane, SDS-polyacrylamide gel and Whatmann paper. Transfer was run for 1 h at 0.33A per SDS-polyacrylamide gel with no more than 25V. After the transfer the membrane was blocked using blocking solution from the Western Breeze Chemiluminescent Anti-Rabbit Kit. Protein bands were detected with polyclonal anti-GFP antibody (1:1250, Invitrogen) and the Western Breeze Chemiluminescent Anti-Rabbit Kit according to the manufacturer's instructions. As the kit uses a secondary antibody fused to the enzyme horseradish peroxidase (HRP) ECL chemiluminescent substrate could be used to detect protein bands and develop with Hyperfilm ECL sheets (Amersham).

3.8.4 Subcellular fractionation

The parasite pellet (collected as described in 3.9.1) was resuspended in hypotonic solution (10 mM TRIS, pH 8.0, 5 mM EDTA, complete protease inhibitor cocktail) and frozen and thawed (while extensive pipetting) three times before 30 min incubation on ice. After 1 h centrifugation at 100,000 x*g* the supernatant containing the cytosolic fraction was collected and the pellet washed once with hypotonic solution. After resuspension in the carbonate solution (0.1 N Na_2CO_3 pH 11.0, complete protease inhibitor cocktail) and centrifugation at 100,000 x*g* for 1 h the supernatant containing the peripheral membrane fraction was obtained. To

collect the integral membrane fraction, the pellet was washed once with carbonate solution and resuspended in PBS 1X containing 1% NP-40, incubated on ice for 10 min and centrifuged at 16,000 x*g*.

3.9 Imaging and microscopy assays

3.9.1 Isolation of schizonts, gametocytes and ookinetes

Purification of schizonts was achieved by placing blood cells collected from infected mice (4-5% Parasitaemia) in culture (RPMI pH 7.3 containing 25% foetal calf serum and 0.5 mL Penicillin/Streptomycin (300U/mL)) and incubating for 24 h at 37°C. Maturity of schizonts was checked with Giemsa staining of blood smears. Schizonts were isolated on a NycoDenz gradient (60% v/v in PBS; stock solution: 27.6% w/v NycoDenz in 5 mM Tris-HCl, pH 7.2, 3 mM KCl, 0.3 mM EDTA), harvested from the interface and washed.

Ookinetes were isolated by incubating infected blood cells (10-15% Parasitaemia) in culture for 24 h at 20°C. Proper formation of ookinetes was checked with live-cell imaging before purifying the cells on a NycoDenz gradient (62.5% v/v in PBS). Ookinetes were harvested from the interface and washed before further usage.

Gametocytes were isolated on a NycoDenz gradient (48% v/v in CLB, coelenterazine loading buffer: PBS, 20 mM HEPES, 20 mM Glucose, 4 mM sodium bicarbonate, 1 mM EGTA, 0.1% w/v bovine serum albumin, pH 7.25), washed and harvested from the interface. Gamete activation was achieved by incubation in ookinete media for 20 min and exflagallation checked with live-cell imaging.

3.9.2 Preparation sporozoites

To obtain *Plasmodium* sporozoites *Anopheles stephensi* mosquitoes were allowed to feed from infected mice (parasitaemia 10 – 15%) for 20 minutes to ingest gametocytes. Mosquitoes were then kept at 20°C for up to 22 days to allow the parasite to grow and develop within the mosquito midgut until mature sporozoites are formed after approximately 14 days. Mosquito guts were dissected after 7, 14 and 21 days and salivary glands additionally after 21 days. After imaging guts were homogenised in PBS 1X to release single sporozoites. Cells were centrifuged for 5 min at 2,500 x*g* and cell pellet was stored at -20°C until further usage.

3.9.3 Live-cell imaging

The expression of the GFP-tagged gene was tested via visualisation of transgenic parasites on a Zeiss AxioImager M2 (Carl Zeiss, Inc) microscope fitted with an AxioCam ICc1 digital camera (Carl Zeiss, Inc). Different parasite stages were isolated as described in chapter 3.9.1 and 3.9.2. Cells were stained with Hoechst (1:1000) for 5 min prior to mounting and ookinetes were additionally stained with 13.1., an antibody detecting the surface protein P28.

3.9.4 Immunofluorescence assay

Isolated parasites were smeared onto poly-L-lysine covered slides and fixed in 4% PFA. Cells were permeabilized in 0.15% Triton-X 100 and blocked in 3% BSA in PBS 1X. Slides were incubated with poly-clonal rabbit anti-GFP (1:250, Invitrogen) or monoclonal mouse H4K20Me3 (1:1000) antibodies in 1% BSA in PBS 1X for 1 h and washed in PBS 1X three times. AlexaFluor 568 labelled anti-mouse (1:1000, Invitrogen) and AlexaFluor 466 labelled anti-rabbit (1:1000, Invitrogen) were used as secondary antibodies and incubated in 1% BSA for 1 h

together with Hoechst. Slides were washed three times in PBS 1X, mounted with Fluoromount-G mounting media and kept in the dark overnight to dry. If necessary, cells were incubated with 13.1 antibody (1:1000 in PBS) prior to blocking.

3.9.5 Superresolution microscopy

Samples were fixed and stained directly on coverslips and later mounted onto microscopy slides. Prior to cell treatment coverslips were coated in poly-L-lysine. Briefly, coverslips were sterilized with HCl for 30 min at room temperature and washed with ultrapure water and 100% Ethanol. Coverslips were then coated in poly-L-lysine and incubated for at least 30 min before washing progressively with 70%, 95% and 100% Ethanol. Parasite samples were then pipetted onto coverslips and kept at 4°C for two days to settle. Finally, coverslips were mounted with Fluoromount-G mounting media.

Superresolution microscopy was performed at a 3D-SIM Zeiss microscope fitted with 405 nm, 488 nm and 568 nm lasers and blue, green, red and far red filters. ZEN Digital Imaging for light microscopy was used for obtaining images. For Fluoromount-G mounted slides water-based oil and the respective objective were used for imaging.

4 Results

4.1 Expression and Localization of the Large Armadillo repeat Protein (LARP)

In order to study the still uncharacterized ARM repeat protein PBANKA_113780, here referred to as LARP, the protein was endogenously tagged with the green fluorescent protein (GFP) at its C-terminal end. Using single homologous recombination, the GFP-tag was inserted downstream of the endogenous *larp* gene, by removing the stop codon (Figure 13A). The endogenous expression of the GFP-tagged protein allows the investigation of the cellular dynamics of the protein's expression pattern and localization during the *Plasmodium berghei* life cycle, by live-cell imaging.

4.1.1 Genotypic analysis confirms successful integration of LARP-GFP

After successful transfection of the LARP-GFP construct, genotypic analysis was used to validate the correct integration of the construct the right genomic location and the expression of a GFP-tagged LARP protein. Diagnostic PCR on isolated genomic DNA from both LARP-GFP and WT (negative control) parasites, using the primers IntT135 and ol492 (as indicated in Figure 13A), confirmed the correct integration. A fragment of the expected size of 1937 bp was amplified in LARP-GFP (Figure 13B), while no amplification was detected in WT parasites. As the primers used for diagnostic PCR are designed to only bind to the integrated GFP-construct and not to the WT genome, these results confirm the successful integration of the LARP-GFP construct.

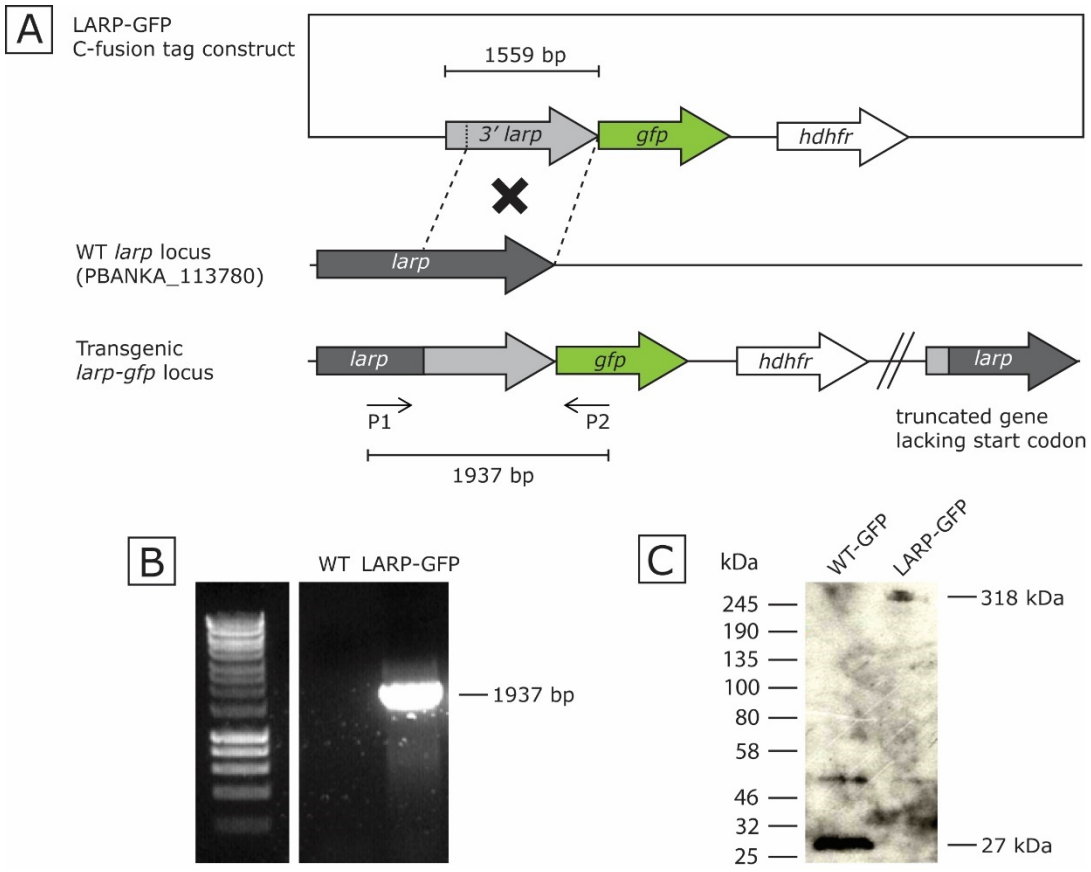


Figure 13| Generation of a *Pb*ANKA line expressing LARP-GFP: (A) Schematic visualisation of the strategy used for endogenous GFP-tagging of LARP at its C-terminal end. The vector construct contains the 3' gene fragment of *larp* directly followed by a gfp sequence and an *hdhfr* cassette in the 3' UTR. Through single homologous recombination the fragment was integrated downstream of the *larp* locus. Arrows indicate the binding sites of primers used for diagnostic PCR (P1: IntT135; P2: ol492). **(B)** Agarose-gel electrophoresis of diagnostic PCR products, amplified using the primers IntT135 and ol492. Amplification of a 1973 bp fragment in LARP-GFP confirms a successful integration. No band was produced in the WT lane, as ol492 is binding to the *gfp* sequence in the inserted construct and is not able to anneal to the WT genome. **(C)**Western Blot analysis confirms expression of a GFP-tagged LARP protein. Anti-GFP antibodies detect band at the expected size of 318 kDa in LARP-GFP lysates.

The correct expression of the GFP-tagged LARP protein was confirmed by Western Blot analysis of asexual blood stage lysates isolated from LARP-GFP and WT-GFP parasites. The anti-GFP antibody detected WT-GFP at the expected molecular weight of 27 kDa (Figure 13C). LARP-GFP was detected at approximately 318 kDa (Figure 13C). This size corresponds to the expected molecular weight of

the LARP-GFP fusion protein (LARP: 291 kDa + GFP: 27 kDa), and is thereby validating the correct expression of the GFP-tagged protein.

4.1.2 LARP is highly expressed throughout the *Plasmodium berghei* life cycle

In order to characterise the cell biology of LARP, its gene expression, both at RNA transcript and protein level, was analysed throughout the whole *Plasmodium* life cycle. The first step of gene expression is the transcription of mRNA. To determine the level of mRNA synthesized in the parasite during the different life cycle stages, qPCR was performed on RNA isolated from WT schizonts, activated gametocytes and ookinetes. As demonstrated in Figure 14 the transcript level of *larp* differs between the life cycle stages. While the transcript is expressed in all life cycle stages investigated, the mRNA level was highest in schizonts. In ookinetes, on the other hand, the lowest *larp* transcription was observed, with an mRNA level less than 1/10 of the level detected in schizonts. However, the results



Figure 14| *larp* mRNA expression in different life cycle stages: *larp* transcript expression was analysed by performing qRT-PCR on RNA isolated from WT-GFP schizonts, activated gametocytes and ookinetes. Expression was normalized to GFP and Arginine-tRNA-ligase (for calculations see Appendix E). Amplification was detected using SYBR green dye. (n=3)

display large error bars, especially in schizonts, which indicates a high variation between the different samples examined.

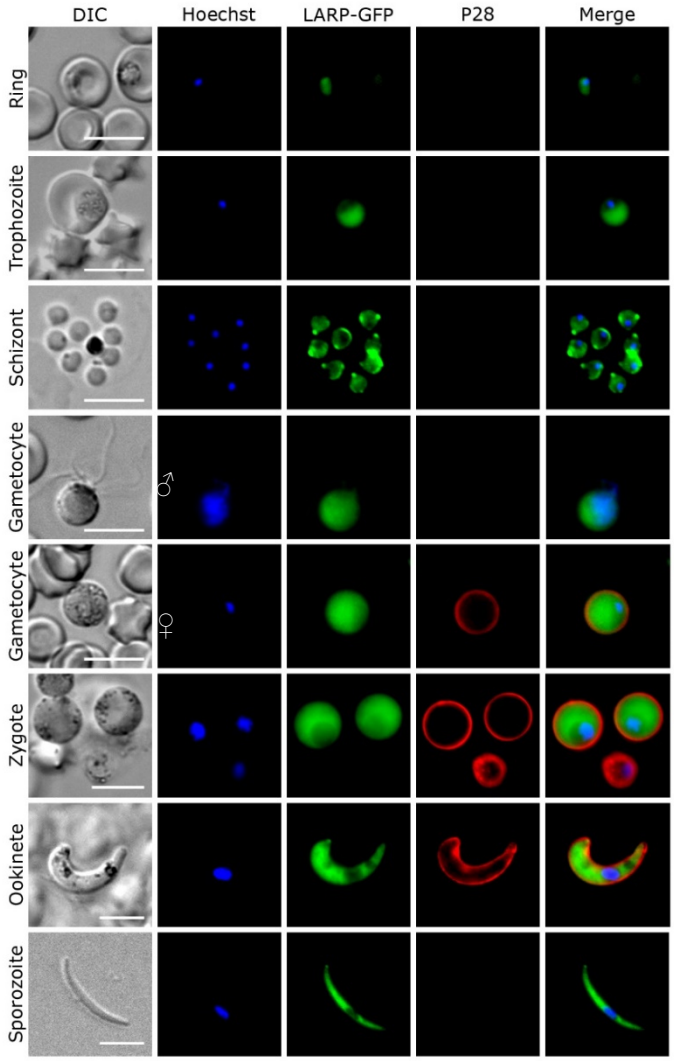


Figure 15| LARP-GFP protein expression throughout the *P. berghei* life cycle: Parasites were isolated at each life cycle stage and revealed by differential interference contrast (DIC). Hoechst was used to detect the nucleus of each parasite and the P28 surface protein of activated female gametocytes, zygotes and ookinetes was detected by the 13.1 antibody. The merge image comprises the Hoechst, GFP and P28 channel. LARP-GFP is expressed throughout the cytosol in every life cycle stage and shows a distinct apical localisation in schizont, ookinete and sporozoite. Scale bar = 5 µm.

The LARP protein expression during various life cycle stages was initially analysed through live-cell fluorescent microscopy of LARP-GFP expressing parasites. Each of the life cycle stages analysed was isolated and stained with Hoechst to mark the parasite nucleus. During this study, liver stage parasites could not be investigated, as the facilities and resources are currently not available in the lab. As Figure 15 demonstrates LARP-GFP is expressed at every stage analysed in the *Plasmodium berghei* life cycle. It is predominantly located in the cytoplasm and shows no nuclear localisation in most stages examined. In the life cycle stages expressing the P28 surface protein (activated female gametocytes, zygotes and

ookinetes), the LARP-GFP (green) and P28 (red) signal can be clearly distinguished and show almost no co-localisation. This indicates that LARP is predominantly expressed in the cytosol and has close to no membrane attachment. Interestingly, the invasive stages (merozoite, ookinete and sporozoite) additionally show a distinct localisation of LARP-GFP, that forms a dot at the very apical end of the parasite. This signal can be clearly distinguished from the cytosolic expression of LARP-GFP and co-localises with the membrane marker P28 in ookinetes.

4.1.3 LARP shows an apical localisation in the invasive stages of *Plasmodium berghei*

Live-cell imaging is an excellent method to analyse the protein expression and cellular dynamics as they take place in the living cells. However, certain compartments cannot be easily investigated when only using live-cell imaging, as the cells might be moving and the fluorescent signal can bleach, while obtaining the results. Therefore, a fixed immunofluorescence assay helps to receive more detailed information and the combination of both imaging techniques delivers a more comprehensive study of the protein expression of LARP-GFP. IFA was performed on the invasive stages of the *Plasmodium berghei* life cycle (schizont/merozoite, ookinete and sporozoite), particularly to confirm and further characterise the apical accumulation of LARP-GFP. Representatives of the obtained imaging results are displayed in Figure 16A. The IFA images correspond with the results received in live-cell imaging: LARP-GFP is predominantly expressed in the cytosol, lacks nuclear localization and forms a distinct accumulation at the apical end of the invasive stages (indicated by the white arrows in Figure 16A).

However, in order to achieve a more detailed study of the apical localisation of LARP-GFP, a higher resolution of the obtained images is necessary. The resolution of light microscopy used in the results described above is limited to approximately half of the wavelength of light used. Therefore, structures that are

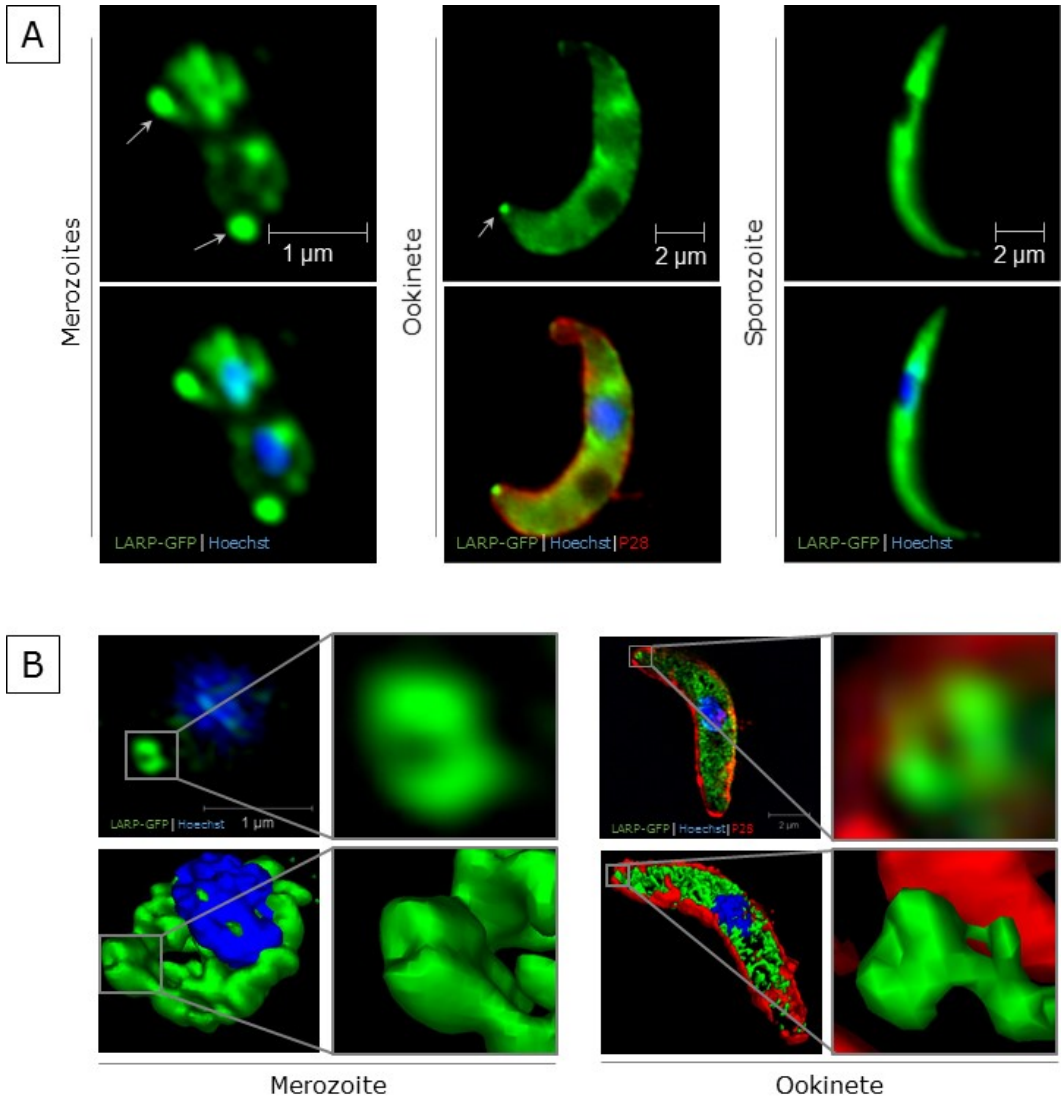


Figure 16| The apical localization of LARP-GFP in invasive stages of *P. berghei*: **(A)** Immunofluorescence microscopy images of merozoites, ookinetes and sporozoites. Anti-GFP antibody was used to detect LARP-GFP, nuclei were stained with Hoechst and P28-antibody (13.1) was used as a membrane marker in ookinetes. Arrows indicate the distinct apical localisation of LARP-GFP. **(B)** Superresolution microscopy images of merozoites and ookinetes. Parasites were prepared and stained as described in **(A)**. Images were obtained with 3D-SIM microscopy and ZEN software was used for image processing and generation of 3D models. The top row displays one representative of Z-stack images obtained. Bottom row demonstrates a 3D re-construction on the basis of obtained Z-stacks. The apical region of the parasite is magnified for each image and displayed on its right hand side.

less than 200 nm to 350 nm apart, cannot be identified as two separate objects. Different methods are known to improve the resolution of microscopy images. Deconvolution microscopy for example, uses computational post-processing to

reconstruct an object from wide-field Z-stack images. Another technique, confocal laser scanning microscopy, uses a tightly focused laser point to scan the sample. Due to a pinhole in the confocal image plane only the light from this focus can pass through. By mapping the detected light the image can be reconstructed afterwards (Schermelleh et al., 2010). However, these methods still hold many disadvantages and limits. In recent years more advanced microscopy methods have been established for bioscientific research. Structured illumination microscopy (SIM) for example, reaches a resolution of approximately 105 nm through the use of illumination patterns. When illuminating fluorescent samples, that contain fine structures, distinct interference patterns (*moiré fringes*) can occur and disturb the detected image. By passing the light through a movable optical grating the samples are illuminated through a striped pattern. When this pattern is applied to the sample in different orientation, a high-resolution image can be re-constructed through computer algorithms using the information obtained (Schermelleh et al., 2010).

Here, structured illumination microscopy (3D-SIM) was used, in order to achieve a much more detailed and in depth study of the apical localisation of LARP-GFP. As can be seen in the images presented in Figure 16B, LARP-GFP produces a strong and clear signal at the apical end of the parasite next to its cytosolic expression. This localisation of LARP-GFP is distinct from and stronger than its cytosolic expression in other areas of the parasite. Due to the high resolution of 3D-SIM, a more detailed image of the apical region is received. The pictures in the upper row of Figure 16B present one layer of the Z-stack images obtained from merozoites and ookinetes. Interestingly, these images demonstrate that LARP-GFP forms a ring-like structure, rather than a dot in the apical region. Within this ring, the strength of the LARP-GFP signal seems to be inconsistent, leading to two dominant and bright signals on either side of the ring. These differences in signal strength can be caused by quenching events during the imaging process. The

bottom row of images in Figure 16B presents the 3D reconstruction of the Z-stack images. While the 3D model of the apical region does indicate the presence of a ring, the structure is not entirely clear in these images. As described above this can be caused by bleaching of the samples during the imaging process.

4.1.4 LARP shows stage-specific developmental profiles during the formation of invasive parasite stages

The results presented above demonstrate, that LARP-GFP shows a distinct localisation at the apical end of the invasive parasite stages, while in all other stages the protein is predominantly cytosolic. To investigate as to when and how this distinct feature develops during the formation of invasive stages, schizogony, sporogony and ookinete formation processes were observed through live-cell imaging. The expression and localisation of LARP-GFP was monitored during these developmental profiles.

To observe the localisation of LARP-GFP during schizogony, different stages of schizont maturation were captured through live-cell imaging. In Trophozoites, as shown in Figure 17, LARP-GFP is solely expressed in the cytosol. However, as soon as the nucleus undergoes mitotic division and the parasite starts to differentiate into a schizont, LARP-GFP accumulates in distinct spots near the plasma membrane. While the schizogony progresses, more distinct dots of LARP-GFP are visible near the plasma membrane until their number corresponds with the merozoites formed during schizogony. Interestingly, until the schizont is fully matured, more dots of LARP-GFP than divided nuclei are present in the cell.

Sporozoites shares a great resemblance with merozoites, due to their similar structure and development. Like merozoites, sporozoites are formed within their mother-parasite (oocyst), though the oocyst is an extracellular parasite, while schizonts are developed within a red blood cell. However, after full

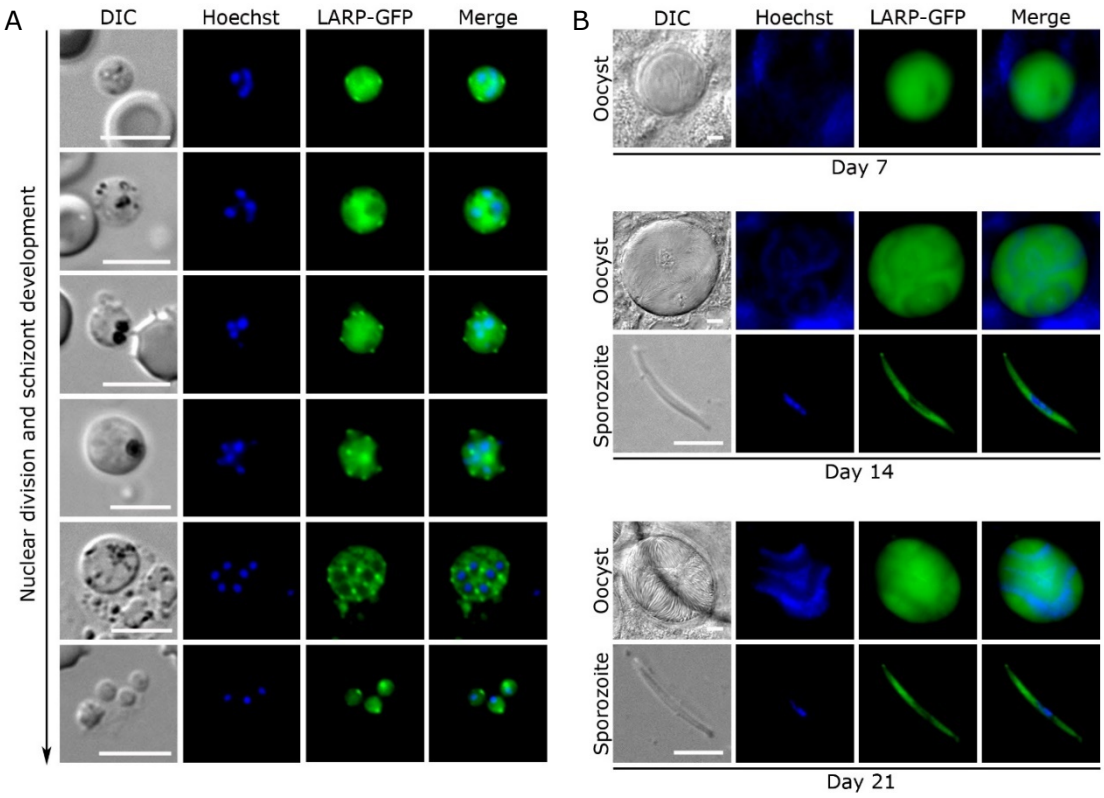


Figure 17| LARP-GFP expression during schizonts and sporozoite development: **(A)** Developmental profile of LARP-GFP localisation during schizont development. Cells were imaged after 4, 8, 16 and 24 h incubation in schizont culture. Hoechst was used to stain the nucleus and parasites were revealed with DIC. Merge image comprises Hoechst and GFP channels. **(B)** Developmental profile of LARP-GFP localization during oocyst development and sporozoite formation. Oocysts were imaged 7, 14 and 21 days after mosquito infection. Sporozoites were mechanically released from oocyst and imaged after 14 and 21 days. Salivary gland sporozoites were analysed after 21 days. Parasites were revealed with DIC and Hoechst was used to stain the nucleus. Scale bar = 5 µm

maturation, the sporozoites are released from the oocyst through a process that is similar to the merozoites egress from erythrocytes (Aly et al., 2009; Gerald et al., 2011). As presented in Figure 17B, LARP is solely expressed in the cytosol while the oocyst is still growing and mitotic division has just commenced (Day 7 post-infection). 14 days post-infection, sporozoites are fully formed but not yet released from the oocyst. During this stage LARP-GFP is still strongly expressed in the cytosol, while no distinct dots formed by LARP-GFP are visible. However, when the mosquito guts are homogenized and sporozoites released from the oocyst through mechanical force, LARP-GFP immediately localises at the apical end

forming the distinctive tip. Within the sporozoites that are still present in the oocyst, this apical signal could potentially be superimposed by the strong cytosolic signal of LARP-GFP in the mother cell. 21 days post-infection, when sporozoites are fully matured, they egress from the oocysts and migrate to the salivary glands. Mature oocyst which were still containing sporozoites showed a similar expression pattern as parasites imaged after 14 days with respects to the LARP-GFP localisation. No distinctive spots of LARP-GFP were detectable in these oocysts. However, the sporozoites invading the salivary glands, as well as sporozoites mechanically removed from oocysts displayed the characteristic localisation of LARP-GFP at their apical end.

While mitotic division in schizont and sporozoite development produces several daughter cells, meiotic division occurs when a zygote develops into an ookinete. The zygote is the only diploid stage in the *Plasmodium* life cycle and through meiotic division it reaches the haploid state during ookinete development (Sinden et al., 1985). Therefore, no cell division occurs and the zygote transforms into retorts, the intermediate stages of ookinete formation, before eventually developing into a motile ookinete. This process can be broken down into 6 stages, as demonstrated in Figure 18 (Janse et al., 1985). The images presented in indicate an expression of LARP-GFP solely in the zygote body at the onset of ookinete formation. Up until stage III of ookinete development the GFP-signal is strongest in the zygote body, much weaker in the forming ookinete and not present at the apical tip. In stage IV the signal starts to distribute throughout the whole retort, now equally strong in both areas of the developing cells. Additionally, LARP-GFP starts to accumulate at the apical end of the parasite, forming the characteristic tip. In stage V LARP-GFP is solely localised in the forming ookinete, the apical signal becomes stronger and more distinct than it was in stage IV. Only a low expression is detectable in the remaining zygote body. As soon as the parasite reaches stage VI and becomes a fully formed ookinete, LARP-GFP displays

a strong apical signal and can be clearly distinguished from the cytosolic expression.

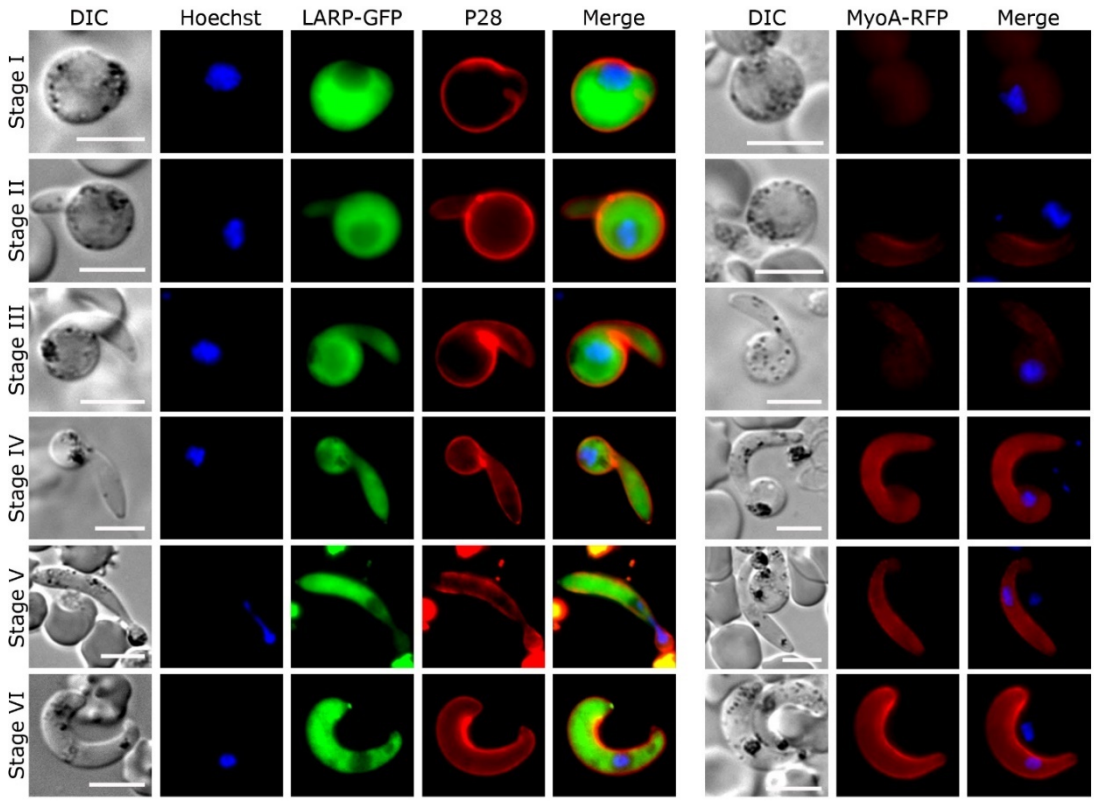


Figure 18| LARP-GFP and MyoA-RFP expression during ookinete development: (A) Developmental profile of LARP-GFP localization during ookinete development. Infected mouse blood was incubated in an ookinete culture for 24 h and imaged in 3 h intervals. Parasites were revealed with DIC, Hoechst was used to stain the nucleus and P28 was visualized and used as a membrane marker. Merged image comprises the Hoechst, GFP and P28 channel. **(B)** Developmental profile of MyoA-RFP localization during ookinete formation. Cells were prepared and visualised as described in **(A)**. Scale bars = 5 µm.

To compare the development of LARP-GFP to a known and well characterised protein that is present in the apical and glideosomal complex, the expression and localisation of MyoA during ookinete development was monitored, as well. MyoA is a protein attached to the membrane and, as a major component of the glideosome, an important factor in the invasion and motility processes of the parasite (Keeley and Soldati, 2004; Meissner et al., 2002). As the images presented in Figure 18 demonstrate, the expression of MyoA-RFP grows stronger while the ookinete is forming. During the stages I to III of ookinete development MyoA-RFP is expressed on a very low level in the forming ookinete and is not

present in the zygote body. With the formation of stage IV and up until the ookinete is fully formed, MyoA-RFP is expressed on a significantly higher level throughout the whole parasite cell. During stage IV to VI MyoA is expressed in the cytosol and attached to the membrane. In the fully formed ookinete, however, the membrane signal of MyoA-RFP is strongest and demonstrated a slight polarity towards the apical end of the ookinete.

4.1.5 Co-localization of LARP with apical proteins and markers

In order to investigate the apical expression of LARP-GFP with respects to known structures, co-localisation assays were performed using different proteins and cellular markers. As already mentioned above MyoA is a major component of the glideosome, involved in invasion and motility and has an apical polarity in the invasive stages of Apicomplexan parasites (Meissner et al., 2002). To achieve an expression of MyoA-RFP and LARP-GFP in the same cell, both parasite lines were

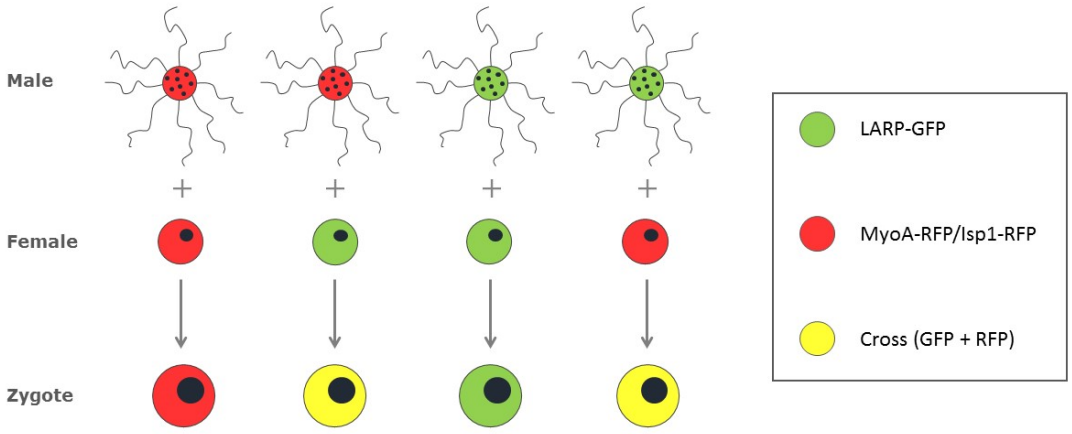


Figure 19| Generating of crossed parasite lines: Schematic demonstration of different zygote-genotypes generated by crossing two different transgenic parasite lines. When male gametocytes fertilize a female gametocyte with the same genotype (expressing the same tagged protein), the phenotype of the zygote remains the same. Fertilization of a female gametocyte with a different genotype (expressing a different tagged protein), results in a zygote containing both mutations and expressing both tagged proteins. Expression of a GFP-tagged and an RFP-tagged protein leads to a yellow signal when the proteins are co-localising.

crossed and the gametocytes activated, either in an ookinete culture or by a mosquito feed. The fertilization between two parasites from different lines generated a certain proportion of zygotes expressing both tagged genes as illustrated in Figure 19. This method allows to detect both proteins with respect to each other in live-cell imaging. As demonstrated in Figure 20A MyoA-RFP and LARP-GFP are co-localised at the apical tip of each invasive parasite stage. LARP-

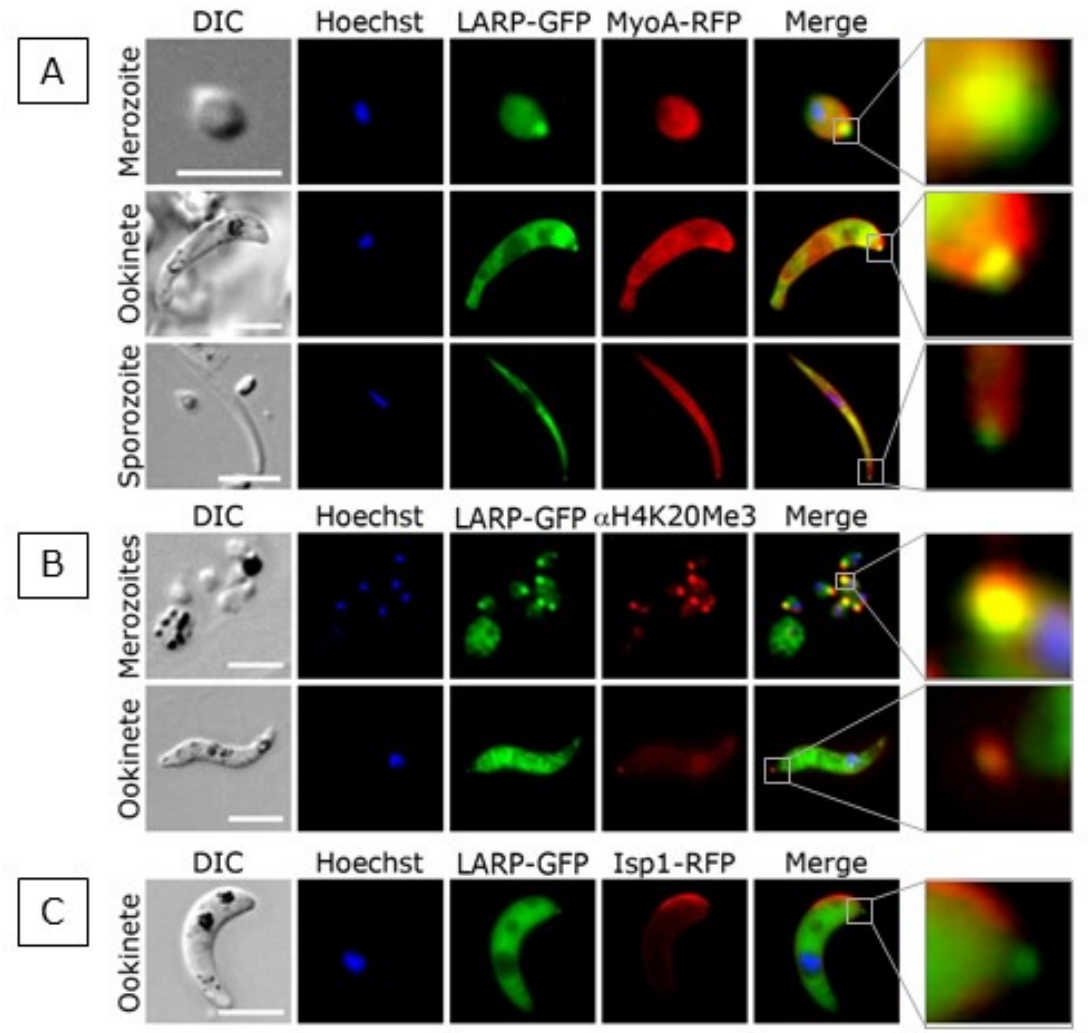


Figure 20| Localization of LARP-GFP in respect to known apical proteins: (A) Live-cell imaging of crossed parasites expressing LARP-GFP and MyoA-RFP in merozoites, ookinetes and sporozoites. Parasites were revealed with DIC and the nucleus stained with Hoechst. **(B)** IFA performed on LARP-GFP merozoite and ookinetes using anti-GFP and anti-H4K20Me3 antibodies. **(C)** Live-cell imaging of crossed parasite expressing LARP-GFP and Isp1-RFP in ookinetes. Parasites were revealed with DIC and nucleus stained with Hoechst. Magnification of apical region is presented on the right hand side of each panel. Scale bars = 5 µm

GFP has the expected predominant cytosolic expression, while MyoA is more restricted to the parasite membrane. At the apical end of the parasite however, the LARP-GFP-signal (green) and the MyoA-RFP-signal (red) are co-localising and therefor producing the detected yellow signal.

Another apical marker in the invasive stages of apicomplexan parasites is the anti-H4K20Me3 antibody that recently has been shown to stain the apical region in *Toxoplasma* and *Plasmodium* parasites (Xiao et al., 2010). This antibody detects methylated lysine residues of proteins and therefore is also an indicator for a post-translational methylation. As presented in Figure 20B, the signal of the anti-H4K20Me3 antibody (red) is clearly staining the apical tip of ookinetes and merozoites, while only producing a weak signal in the cytoplasm. This apical signal is fully co-localizing with the apically expressed LARP-GFP in merozoites and ookinetes, as the images presented in Figure 20B demonstrate.

In a third co-localisation assay, parasites expressing LARP-GFP and Isp1-RFP were examined in live-cell imaging. Isp1 is an IMC sub-compartment protein and an early marker for apical polarity during ookinete development in *Plasmodium berghei* (Poulin et al., 2013). Isp1-RFP and the characteristic dot of LARP-GFP are both located in the same area of the ookinete (Figure 20C), confirming the apical localisation of LARP-GFP. However, the proteins are adjacent to each other but are not co-localising. Isp1-RFP can be detected at the membrane in the apical region, with a predominance at one side of the parasite. At the very apical tip though, where LARP-GFP is localised, Isp1-RFP is not expressed.

4.2 Biochemical analysis

4.2.1 Subcellular fractionation confirms solubility and partial membrane attachment of LARP

During live-cell imaging and the immunofluorescence assays performed on the invasive stages of *Plasmodium berghei*, the apical accumulation of LARP-GFP seemed to be attached to the parasite's membrane, as it was co-localizing with the P28 signal in ookinetes. This partial membrane attachment of LARP-GFP was confirmed by subcellular fractionation assays in schizonts and ookinetes. Through the use of a hypotonic buffer the cytosolic proteins can be separated from membrane proteins, which again are isolated through the use of a carbonate solution (described before in Beck et al., 2010)

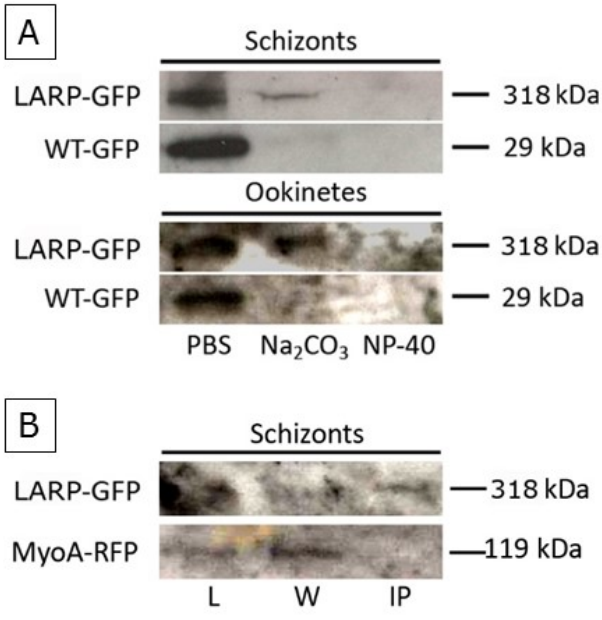


Figure 21| Biochemical analysis of LARP-GFP: (A) Subcellular fractions were isolated from schizonts and ookinetes using a hypotonic lysis buffer (cytosolic fraction), a carbonate lysis buffer (peripheral membrane) and Np-40 (integral membrane). Proteins were detected by Western Blot using anti-GFP antibodies. WT-GFP was used as a control. **(B)** GFP-Immunoprecipitation was performed on parasites expressing LARP-GFP and MyoA-RFP. Protein content in lysate fraction (L), wash fraction (W) and immuno-precipitated fraction (IP) was detected via Western Blot using anti-GFP and anti-RFP antibodies.

Figure 21A shows the results of the subcellular fractionation assays in schizonts and ookinetes; both stages gave similar results. LARP-GFP can predominantly be found in the cytosolic fraction, which correlates with the results

obtained from live-cell imaging. However, a small amount is in fact present in the peripheral membrane fraction in both schizonts and ookinetes. The WT-GFP control was only detected in the cytosol, which confirms that there was no contamination between the two fractions.

4.2.2 Interaction of LARP with apical proteins was not validated through proteomic analysis

ARM repeat domains are known for their various protein-protein interactions. Because of these interactions ARM repeat proteins are involved in various cellular mechanisms. In order to investigate possible interaction partners of LARP, immunoprecipitation assays and proteomic analysis were performed on LARP-GFP.

The attempt to identify interacting proteins after the immunoprecipitation of LARP-GFP from schizonts through mass spectrometry gave inconclusive results. No proteins were detected in the immuno-precipitated fraction. However, a silver staining assay of these samples revealed several proteins to be present in the immuno-precipitated fraction (results not shown here; due to other samples on the same gel with a lower concentration, the respective lanes had to be overexposed), indicating potential interaction partners of LARP. However, without mass spectrometry these proteins could not be identified. In order to investigate one specific interaction instead of the complete interactome, immunoprecipitation was performed on the parasite line expressing the two proteins LARP-GFP and MyoA-RFP. The results obtained from the Western Blot are presented in Figure 21B. LARP-GFP was detected in the protein lysate fraction, the wash fraction and the final immunoprecipitate where its band is strongest and clearest. MyoA on the other hand, could only be detected in the lysate and wash fraction, but not in the precipitate.

It is important to note that, due to the limited time, this experiment was only performed once and under difficult conditions. When crossing the MyoA-RFP and LARP-GFP parasite different lines are produced expressing either LARP-GFP, MyoA-RFP or both proteins (crossed). In this experiment the proportion of only MyoA-RFP expressing parasites was significantly higher than the other two. This low amount of parasites expressing both tagged proteins caused difficulties for detecting LARP-GFP and the pulldown of interaction partners.

4.3 Functional Analysis

4.3.1 LARP is likely to be essential in erythrocytic stages

To identify the potential role and function of LARP within the *Plasmodium* parasite's life cycle a gene knockout was generated. Studying the impact of LARP-depletion on the parasite's viability and phenotype could allow a first identification of mechanisms the protein might be involved in. Through double homologous recombination the *larp* gene was deleted, as indicated in Figure 22. However, no drug resistant parasites could be obtained in pyrimethamine treated mice after the

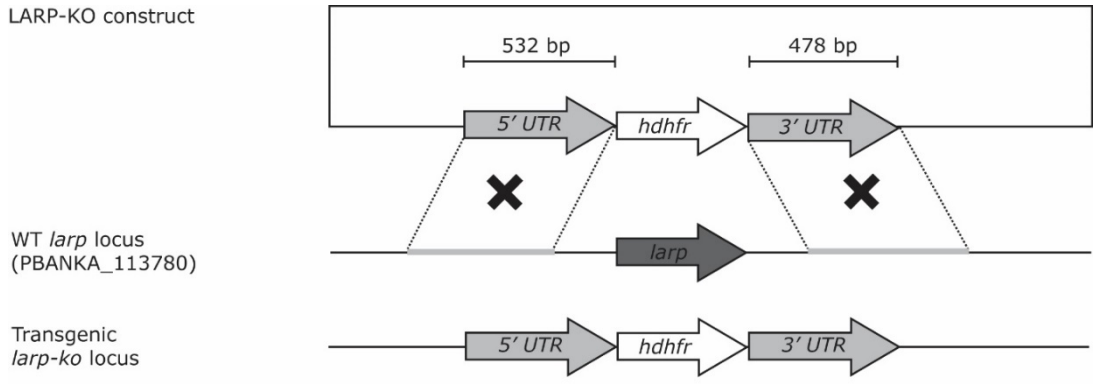


Figure 22| Schematic representation of the gene knockout strategy: The knockout construct contains an *hdhfr* cassette flanked by the 5' UTR and 3' UTR of the *larp* gene. Through double homologous recombination the *hdhfr* cassette is inserted into the *Plasmodium* genome and replacing the *larp* gene sequence.

transfection. For confirmation of these results the experiment was repeated 3 times, but parasites were not viable each time.

4.3.2 LARP could not be disrupted by the tetracycline-repressible transactivator system

Since no viable parasites could be obtained after the complete gene knockout of *larp*, a different system to generate a conditional knockdown was approached. Recent research (Pino et al., 2012) has introduced a tetracycline-repressible transactivator system in *Plasmodium berghei* research. In this system the endogenous target gene is controlled by a minimal promoter, that is regulated by a TetO7 operator sequence. This TetO7 operator sequence is bound and triggered by the tetracycline transactivator (tTA). tTA is a fusion protein of TetR (tetracycline repressor found in *Escherichia coli*) and VP16 (trans-acting protein found in the herpes simplex virus). The transactivator sequence is incorporated into the genome and controlled by the endogenous promoter of the target gene. Therefore, under normal conditions the transactivator is expressed in the same rate as the target gene and triggers the expression of the target gene in that very rhythm. To generate a conditional knockdown system, tTA is composed to be controllable by Tetracycline or its derivatives. The addition of anhydrotetracycline (ATc) for example, inhibits the binding between tTA and the TetO7 operator, hence the target gene can no longer be expressed (Figure 23A).

To achieve a conditional knockdown for LARP using the described system, the necessary components (*trad4* transactivator sequence, *hdhfr* cassette, *TetO7* operator sequence, minimal promoter sequence and polyhistidine-tag) were integrated upstream of the *larp* gene through double homologous recombination, as described in Figure 23B. However, after more than three transfections, no pyrimethamine resistant parasites were obtained. Hence further analysis was not possible for this approach.

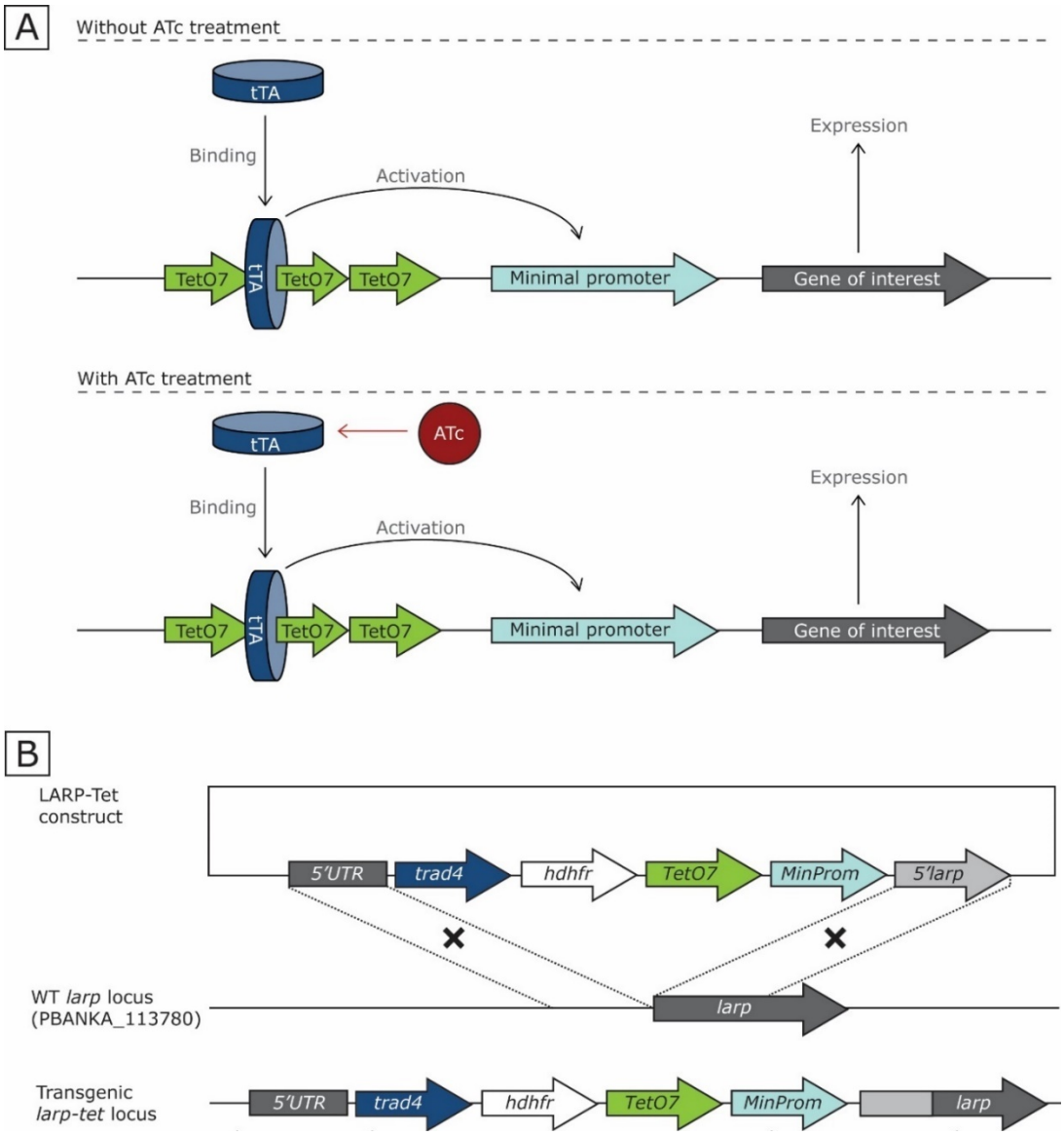


Figure 23| Generation of a tetracycline-repressible transactivator system: (A) Schematic portrayal of the conditional knockdown system. The gene of interest is regulated by a minimal promoter which is controlled by the TetO7 operator sequence. This operator is bound and activated by a transactivator (tTA) that is controlled by the endogenous promoter of the gene of interest. Under normal conditions the gene is expressed in its WT pattern. The presence of anhydrotetracycline (ATc) inhibits the transactivator and gene expression is stopped. **(B)** Schematic representation of the strategy used to generate the conditional knockdown line. Through double homologous recombination a construct containing the *trad4* transactivator sequence, the *hdhfr* cassette, the TetO7 operator sequence, the *minimal promoter* sequence and the polyhistidine-tag was integrated upstream of the *larp* gene sequence.

4.3.3 Conditional expression of LARP shows no effect on parasite viability and development

As all previous attempts to deplete LARP for a functional analysis have been unsuccessful, a promoter swap technique was used for further functional analysis. Through double homologous recombination, as described in Figure 24A, an *ama1* promoter sequence was inserted upstream of the *larp* gene locus. Through this technique *larp* expression is controlled by the AMA1 promoter, which has been used before in the conditional knockdown of CDPK1 and MyoA (Sebastian et al., 2012; Siden-Kiamos et al., 2011). In the following life cycle stages (gametocytes and ookinete), however, AMA1 has a significantly lower transcript level. Thereby, a high expression of LARP is provided in the asexual blood stages, securing the viability of the freshly transfected schizonts. In the later stages (gametocyte and ookinete), on the other hand, LARP would be downregulated to a significantly lower level, allowing a phenotypic analysis of this conditional knockdown.

After successful transfection of the construct the integration of the AMA1 promoter sequence was tested using a diagnostic PCR (Figure 24B). To confirm the correct integration of the 5' UTR sequence the primers INT5'PTD011 (forward) and INT5'PTD (reverse) were used as indicated in Figure 22B. The fragment amplified by these two primers in LARP-PTD has the expected size of 1602 bp and was not produced in the WT control. The integration of the 3' fragment was controlled using the primers 3'INTAMA1 (forward) and INT3'PTD011 (reverse), annealing to the regions indicated in Figure 22B. The expected fragment of 993 bp was amplified from the mutant genomic DNA but not in the WT control. To determine the amount of WT parasites within the LARP-PTD parasite line, that have survived the drug selection, a diagnostic PCR using the primers INT5'PTD011 and INT3'PTD011 was performed. Both primers anneal to a sequence in the parasite's wild-type genome and therefore amplify a fragment in both parasite

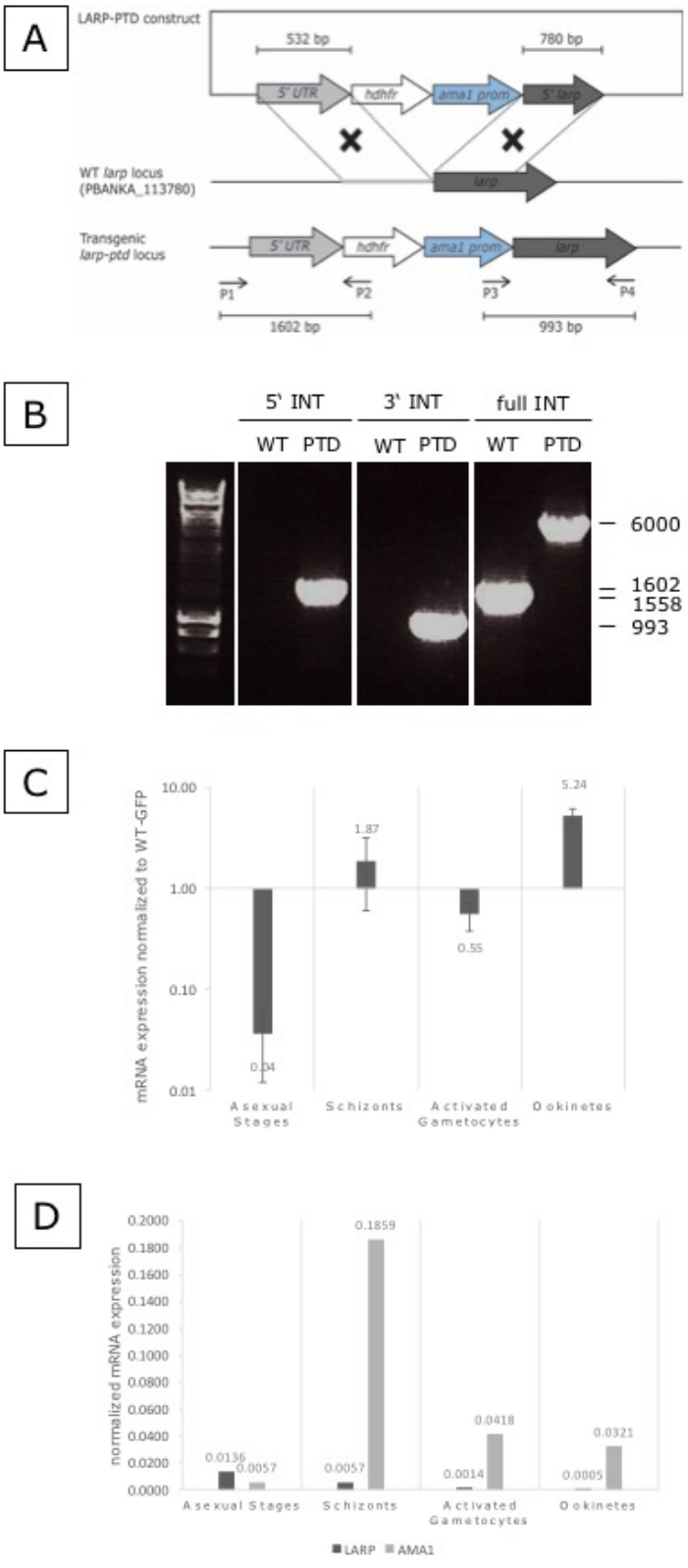


Figure 24| Genotypic analysis of the promoter swap: (A) Schematic representation of the strategy used to generate the promoter swap. Construct contains *hdhfr* cassette and *ama1* promoter sequence flanked by 5' UTR and 5' gene region of *larp*. Construct was integrated into the genome through double homologous recombination. Arrows indicate primer binding sites used for diagnostic PCR (P1: INT5'PTD011; P2: INT5'PTD; P3: 3'INTAMA1; P4: INT3'PTD011). **(B)** Diagnostic PCR was performed to confirm successful integration of the promoter swap construct into the parasite genome. Primer pairs INT5'PTD011/INT5'PTD (5' integration) and 3'INTAMA1/INT3'PTD011 (3' integration) were used to amplify integrated fragments. WT DNA was used as a negative control. **(C)** QRT-PCR was performed on LARP-PTD RNA isolated from asexual blood stages, schizonts, gametocytes and ookinetes, to analyse *larp* gene expression under the control of the AMA1 promoter in reference to the WT expression. Arginine-tRNA-ligase and Hsp70 were used as reference genes. **(D)** QRT-PCR was performed on WT-GFP RNA, showing the transcript profile of LARP and AMA1 in WT parasites.

lines. In WT DNA a band of approximately 1558 bp was amplified, which

corresponds to the expected size of the fragment in the WT genome. In LARP-PTD the band was detected at approximately 6000 bp, the expected size of the integrated fragment. No WT band was detected in the LARP-PTD lane, indicating a clean parasite line.

The changes in the mRNA level caused by the conditional expression of *larp* under the control of the AMA1 promoter, were analysed using qRT-PCR. The *larp* transcript expression in asexual blood stages, schizonts, activated gametocytes and ookinetes was investigated. Arginyl-tRNA-ligase and Hsp70 were used as reference genes and the received CT-values analysed using the $\Delta\Delta C_T$-method (Zhang et al., 2015) as described in Appendix E. This method allows to compare the mRNA level in the LARP-PTD mutants to the transcript level in a WT control. Therefore, the results presented in Figure 24C indicate the expression of *larp* mRNA in the mutant parasites relative to the WT control. The value 1 resembles an equal level of mRNA expression in WT and LARP-PTD. A value higher or lower than 1 indicates an upregulation or downregulation of the *larp* transcript, respectively. The expression of *larp* is downregulated in asexual blood stages and gametocytes and not significantly upregulated in schizonts. By securing the high expression of LARP, the schizonts are fully viable and can proceed in the *Plasmodium* life cycle. Interestingly, LARP expression is also and very unexpectedly upregulated in ookinetes. Due to its low transcript expression in ookinetes, AMA1 is used as a common promoter for this technique (Sebastian et al., 2012; Siden-Kiamos et al., 2011). However, these results indicate that endogenous LARP is expressed at a much lower level than AMA1 at the ookinete stages and hence is upregulated when under the control of the AMA1 promoter. A qPCR analysis of the expression of LARP and AMA1 in WT ookinetes (Figure 24D) confirms this hypothesis and shows clearly, that in ookinetes the *larp* transcript is lower expressed than *ama1*.

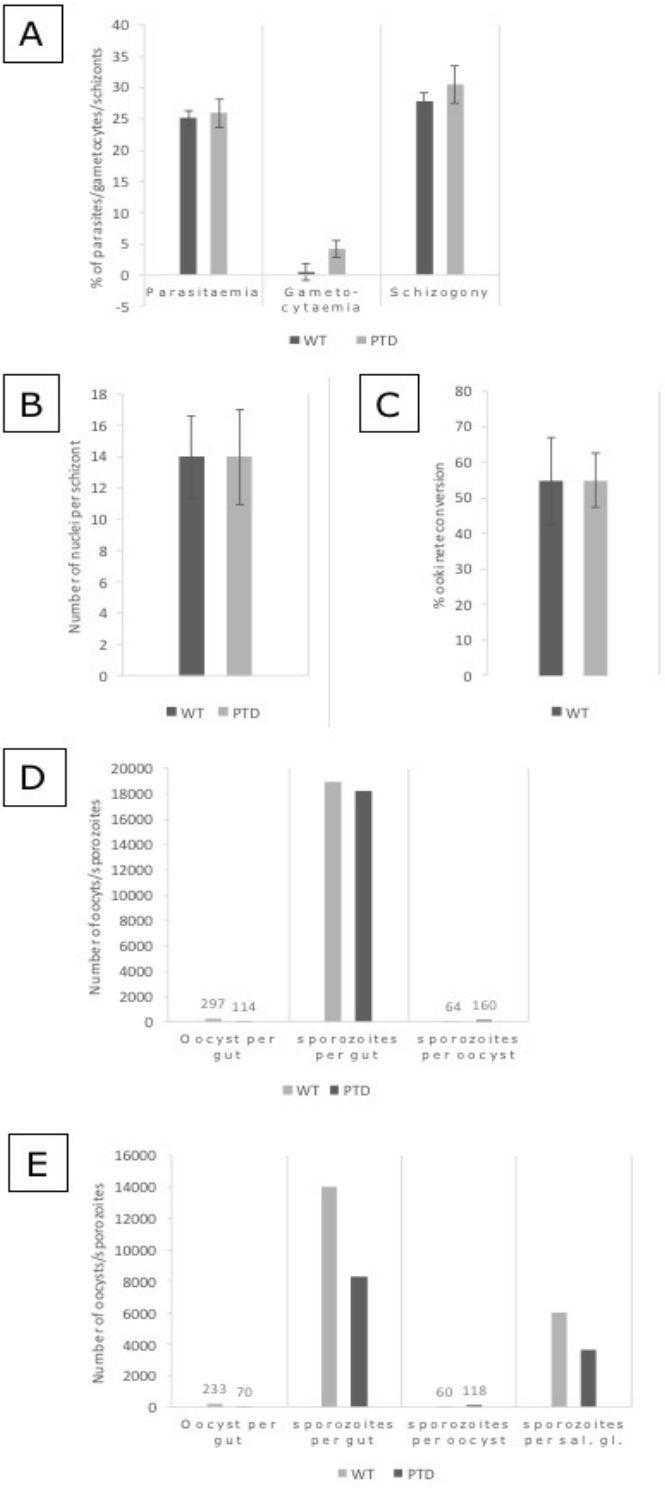


Figure 25| Phenotypic analysis of the promoter swap: (A) Parasite development after the conditional expression of *larp*. Parasitaemia is the percentage of red blood cells invaded by parasites. Gametocytaemia is determined by the number of parasites that have developed into gametocytes. Schizogony expresses the percentage of asexual blood stages that are fully matured schizonts. Parasites were observed using Giemsa stained slides. Error bars are ± S.D. (n=4). **(B)** Average number of nuclei in fully matured schizonts assessed through Giemsa stained blood smears. Error bars are ± S.D. (n=4). **(C)** Ookinete conversion was determined in WT and LARP-PTD after incubation of blood for 24 h in ookinete culture. Ookinetes were visualised with fluorescent microscopy using the 13.1. antibody. Error bars are ± S.D. (n=4). Ookinete and sporozoite development 14 **(D)** and 21 **(E)** days post-infection. Oocyst were visualised through fluorescent microscopy, sporozoites through light microscopy. (n=1)

To determine the effects of the transcript downregulation in asexual blood stages and upregulation in ookinetes, a phenotypic analysis was performed on different life cycle stages. The results of these experiments are presented in Figure

25. Parasitaemia and gametocytaemia of infected mouse blood were analysed on Giemsa stained blood smears. As presented in Figure 25A no significant differences between LARP-PTD and WT parasites are observed for these values. Schizont development was examined by determining the percentage of asexual blood parasites that have developed to fully matured schizonts. Blood smears of LARP-PTD and WT displayed a similar percentage and no significant alternations were detected. In average each schizont contained 14 nuclei in WT and LARP-PTD parasites Figure 25B, indicating no effects on schizont development. To assess the ookinete conversion in LARP-PTD, freshly collected blood from infected mice was incubated in an ookinete culture for 24 h. Ookinetes were fully formed after 24 h and the conversion rate was equal to WT parasites (Figure 25C).

In order to analyse sporozoite development, mosquitoes were allowed to feed from LARP-PTD and WT infected mice. 14 and 21 days after the infection, mosquitoes were dissected and sporozoite formation was analysed (Figure 25D-E). Mosquito gut infection by LARP-PTD parasites was slightly decreased, as indicated by the lower number of oocysts detected in the mosquito gut 14 and 21 days post infection. However, sporozoite formation within the oocyst was not affected. The low numbers of LARP-PTD sporozoites invading salivary glands 21 days after the infection correspond to the number of oocysts in the mosquito gut and are likely to be caused by the decreased gut infection.

5 Discussion

The family of Armadillo repeat proteins plays essential roles in eukaryotic cells across the animal kingdom and is involved in many different cellular and biochemical mechanisms. Their importance has been well studied amongst many eukaryotes, where they were identified to be involved in cell signalling mechanisms, gene regulation, cell motility and stability as well as cellular transport mechanisms (Coates, 2003; Tewari et al., 2010) and are therefore likely to be involved in various diseases. These proteins receive their versatile interaction abilities from the superhelix, formed by Armadillo tandem repeats, which offers an excellent platform for different protein-protein interactions (Peifer et al., 1994). These properties make ARM repeat proteins essential in various cellular functions and signalling processes and hence are of great interest for future research.

The localisation and role of ARM repeat proteins has hardly been investigated in Apicomplexan parasites and especially in the *Plasmodium*, even though these parasites still hold a severe threat to humans (WHO world report, 2015). Based on the diverse and pivotal role of ARM repeat proteins in other eukaryotic cells (Coates, 2003) this study was undertaken to understand their function within the *Plasmodium* species. Ten ARM repeat proteins have been identified in apicomplexan parasites and five of these are specific for the phylum (Tewari et al., 2010), which is why they are of special interest. As they do not show any resemblance to proteins within the human host, they could act as excellent drug or vaccine targets. Besides studies on one of those proteins (ARO) in *Toxoplasma gondii*, another important apicomplexan pathogen (Beck et al., 2013; Mueller et al., 2013), the function and importance of these apicomplexan-specific ARM repeat proteins during the parasite life cycle have not been studied.

This study characterizes the LARP protein (PBANKA_113780), one of the five phylum-specific ARM repeat proteins, for the first time in its localization and function in *Plasmodium berghei*. By highlighting the importance of this protein, this work sheds further light onto ARM repeat proteins in apicomplexan parasites, as their proper functioning could be pivotal for the parasite's survival. Hence, their full characterisation could hold valuable information for drug discovery and treatment of the malaria disease.

5.1 Expression and Localisation of LARP

Recent studies of genome wide transcription levels have identified the *larp* gene to be expressed in every life cycle stage of *Plasmodium berghei*, with its highest mRNA level in schizonts (Otto et al., 2014). These results were confirmed during this project. While the *larp* mRNA is predominantly synthesized in the schizont stage, its transcription reaches a low point in ookinetes. The results obtained through qRT-PCR however, display relatively large error bars, indicating a high variation between the different samples measured. Repetitions of this experiment with freshly made RNA samples, could provide more accurate results. Nevertheless, the overall trend of the transcription profile of *larp* and the differences between the life cycle stages are clearly indicated in this study.

Similar to the gene transcript the GFP-tagged LARP protein is expressed throughout the *Plasmodium berghei* life cycle. LARP-GFP is predominantly present in the cytosol, which can be observed in every life cycle stage. The significant difference between the schizont and ookinete expression level, that was clearly detected for mRNA transcription, was, however, not observed for the LARP protein. Fluorescent live-cell imaging, though, is a qualitative and not quantitative analysis of a protein. Therefore, exact information on the protein expression level cannot be concluded from this study. A quantitative evaluation of the protein expression could be obtained through further experiments, such as two-dimensional gel

electrophoresis and quantitative mass spectrometry. However, the fluorescent GFP signal in ookinetes is strong and easy to detect, hence, a significant level of LARP-GFP has to be expressed in ookinetes. A high protein level, despite a low mRNA transcription rate could for example be caused by a high stability of the protein and a lack of degradation, so that the protein accumulates within the cell. The stability of the protein could potentially be altered by the GFP tag. In mammalian cells GFP has a half-life of 26 h (Corish and Tyler-Smith, 1999). This high stability of its tag could inhibit the degradation of LARP and cause an accumulation of the protein throughout several life cycle stages. Wether or not the protein is synthesised *de novo* in the ookinete stage or remains stable in the cell over several life cycle stages could be determined through further experiments. FRAP (Fluorescence Recovery after Photobleaching) is a well-established method used with *Plasmodium* parasites (e.g. Matz et al., 2015). With a short flash of light, live parasites can be completely bleached and observed through a fluorescent microscope. The generation of a new fluorescent signal then indicates the time point of *de novo* LARP-GFP translation and synthesis. Further analysing the transcription and translation rates of *larp* in different life cycle stages, could explain the different expression levels between the life cycle stage and might indicate if these are connected to the function LARP has in schizonts and ookinetes.

In addition to the predominant cytosolic expression, LARP-GFP is localising to the apical end of the invasive parasite stages (merozoite, ookinete, sporozoite). LARP-GFP forms a distinct dot, that is clearly distinguished from its cytosolic expression and located in the same area as the invasion and motility machinery of the *Plasmodium* parasites. Interestingly, the apically expressed LARP-GFP is also co-localising with the membrane marker P28 in ookinetes. This could indicate a potential membrane attachment that will be discussed later on. Super-resolution microscopy of the apical region in merozoites and ookinetes, revealed that LARP-GFP is assembled in a ring-like structure at the apical tip rather than a clear dot.

This composition of LARP-GFP bears a strong resemblance to the apical polar ring (APR), one of the organelles forming the apical complex, that is essential for invasion and motility of the parasite. The apical ring is one of the three microtubules organizing centres (MTOCs) in the *Plasmodium* parasite and therefore a major site for microtubule nucleation that is organised in this characteristic ring-like structure. Not much is known about the composition and genesis of the APR; these results, though, propose LARP as a likely candidate for localising to the apical ring. Further experiments are needed to confirm this theory. For example, the co-localisation between LARP-GFP and the apical ring can be validated through the use of APR markers (e.g. RNG1 or RNG2 (Katris et al., 2014; Tran et al., 2010)) in immunofluorescence assays. As the APR is an MTOC, it can also be visualized by using an anti-γ-tubulin antibody, offering another opportunity to study the localisation of LARP in regards to the APR. Following these experiments, a microtubules interaction assay (e.g. co-immunoprecipitation of LARP and tubulin (Takemae et al., 2013)) would be of high interest, as it could identify a direct interaction between LARP and the APR or rather the microtubules emerging from this centre.

The localisation to and interaction with the APR is further supported by the developmental profile of invasive stages. Tracing of LARP expression and accumulation during schizogony revealed a re-localization of LARP-GFP during schizont development. While LARP is expressed solely cytosolic during the trophozoite stage it starts to accumulate in distinct spots near the plasma membrane as soon as the nucleus undergoes mitosis. These spots multiply during the stages of schizogony, until there is one dot per nucleus present in the mature schizont. The apical complex and especially the APR as an MTOC play an essential role during cell division. It has been shown in *Toxoplasma gondii* that the apical ring protein 2 (RNG2) is the first protein to be recruited to the centrosomes after their duplication (Katris et al., 2014). This strongly indicates that the assembly of

the new apical complex is one of the earliest events in cell replication. In fact, during schizogony in *Plasmodium* the APR and other cytoskeletal components are first to be assembled while the nucleus is still dividing and lead to the formation of cleavage furrows, in which the developing merozoite assembles its organelles (Francia and Striepen, 2014). In correspondence to this model, the number of spots formed by LARP is not synchronized to the number of nuclei already produced. Until the schizont is fully mature there are always more LARP dots than nuclei present. This agrees with the APR being assembled early in schizogony to induce the formation of merozoite buds, rather than being recruited to the apical end when the buds are already formed. The fact that LARP starts to accumulate before the daughter cells are fully formed and its possible connection to the APR, could also indicate a possible involvement in cell division.

Sporozoite share many structural and cell biological similarities with merozoites, including their developmental process. Though their mother parasite, the oocyst, is an extracellular parasite, the cell division, maturation and parasite egress are likely to follow a similar pattern as the one observed in merozoites: the apical polar ring and microtubules get assembled before the nucleus is fully divided to create and stabilise the buds, later forming the sporozoites. Like merozoites, fully matured sporozoites that have egressed from the oocyst 21 days post-infection show the distinct apical localisation of LARP-GFP. During the development of these sporozoites, LARP-GFP displays a strong cytosolic expression in the oocyst. Distinct accumulations of LARP-GFP near the oocyst periphery or at the apical end of forming sporozoites, on the other hand, are not visible. However, the high number of sporozoites within a single oocyst and the strong cytosolic signal could superimpose the distinct spots of LARP-GFP. Single sporozoites mechanically released from oocysts 14 days after the gut infection do display the apical localisation of LARP-GFP. This indicates an apical presence of LARP-GFP before the

full maturation of the sporozoites, despite the fact that it cannot be seen in the developing oocyst.

While sporozoites and merozoites develop through mitotic division within their mother cell, ookinetes, the third invasive stage of *Plasmodium berghei*, develop from a zygote that undergoes transformation into different retort stages. The organelles of the apical complex, including the apical polar ring, are synthesized *de novo* and get located to the apical region during ookinete formation (e.g. Counihan et al., 2013). LARP-GFP follows a similar pattern during this developmental process. While the protein is solely expressed in the cytosol during the early retort stages, it gets assembled at the apical tip midway through the formation process and grows stronger until the ookinete is fully matured and motile. Similar to the observed LARP-GFP, the glideosomal complex is assembled in the cytosol and then gets attached to the apical tip during ookinete development (Keeley and Soldati, 2004). Additional to LARP-GFP, this study has monitored the expression of MyoA, a major component of the glideosome and driver of parasite motility. In the non-motile zygote and early retort stages, MyoA is not expressed. With the onset of stage III of ookinete formation MyoA is expressed mainly in the cytosol of the parasite, which is likely to correlate with the time point of glideosome assembly in the cytosol. Towards the final stages of ookinete formation, when the glideosome is attached to the apical membrane, MyoA is predominantly expressed at the membrane of the ookinete and shows a slight apical polarity towards the location of the invasion and motility machinery.

LARP-GFP and MyoA-RFP, not only display a similar developmental profile during ookinete formation, but they also co-localise at the apical tip of all three invasive stages of *Plasmodium berghei* (merozoite, ookinete, sporozoite). Taken together these results strongly indicate a possible connection of LARP-GFP to the invasion and motility machinery of *Plasmodium berghei*, especially to the apical polar ring and the glideosome. However, more experiments have to be performed

for full confirmation and a detailed understanding of LARP localisation. The protein's localisation in reference to the apical polar ring can be characterised through IFA using APR markers such as the protein RNG1 or RNG2. Analysing these samples through super-resolution microscopy could give detailed results on the spatial connection of LARP and the APR. The co-localisation of LARP with the glideosome could further be characterised through the fluorescent detection of other glideosomal proteins (actin, GAP45/50) in immunofluorescence assays. The early assembly of LARP-GFP during the mitotic division processes in schizogony and sporogony together with the localisation to the apical polar ring could also be an indicator for a possible involvement of LARP in cell division processes. However, to clearly determine a role of LARP in cell division or invasion and motility of the *Plasmodium berghei* parasites, further experiments for a functional analysis are needed, as discussed later on.

5.2 Biochemical properties and interaction partners

The high number of ARM repeats within the LARP protein are a strong indicator that LARP could be interacting with various proteins and therefore be involved in many cellular processes. Identifying the interaction partners of LARP is an essential step towards understanding its biochemistry and function. However, the proteomic approaches undertaken in this study were unsuccessful in detecting LARP interacting proteins.

The immunoprecipitation performed in this study has pulled-down interacting proteins, as indicated by silver staining analysis. However, the following mass spectrometry analysis failed to identify significant interaction partners. As in a majority of these experiments only the bait protein was detected through mass spectrometry, while the silver staining clearly indicated interacting

proteins. It can therefore be assumed that these results are caused by a methodological or technical error, rather than a lack of interacting proteins. These errors have to occur after the pull-down experiment was performed, for example during the transportation of the protein samples or during the mass spectrometry itself.

While the study of the general interactome of LARP has been without specific results, the co-localisation of LARP-GFP with MyoA, observed earlier in this study through live-cell imaging, suggests a possible interaction between these two proteins. The performed immunoprecipitation of LARP-GFP, however, detected MyoA in the flow-through fraction, while LARP was, as expected, detected in the immuno-precipitate. The inability to pull-down MyoA together with LARP-GFP indicates that these two proteins, despite their co-localisation, may not be interacting. Due to the time limit, this experiment was only performed once and has to be repeated for confirmation. However, MyoA is only one of possible interaction partners located in the apical region of invasive *Plasmodium berghei* stages. Further experiments are essential to identify specific proteins interacting with LARP. There are different techniques that have been established in recent years to screen the whole interactome of a particular protein in *Plasmodium berghei*. BioID, for example, is a protein interaction assay that has been proven to be successful in apicomplexan parasites in recent research (Chen et al., 2015). By fusing the protein of interest to a mutated biotin ligase (BirA from *E. coli*), the protein is enabled to biotinylate proteins in close proximity to it. Biotinylated proteins can then be isolated through a pull-down and identified afterwards through mass spectrometry (Roux et al., 2013). Furthermore, the interaction of LARP with specific components of the glideosomal and apical complex can be tested. As mentioned before, an actin co-sedimentation assay with rabbit skeletal actin, for example, can assess the actin binding ability of LARP *in vitro* (Mehta and Sibley, 2010).

Though the detection of LARP interacting proteins was not successful during this study, the biochemical analysis did indicate a potential post-translational modification of LARP. The subcellular fractionation assay performed has confirmed the predominant cytosolic expression of LARP-GFP in merozoites and ookinetes. However, a small proportion of the protein was detected in the peripheral membrane fraction of the assay. These results indicate a partial membrane attachment of LARP-GFP in merozoites and ookinetes, as it has already been suspected for the apically localised protein, which is co-localising with the P28 membrane marker in ookinetes. LARP does not possess a transmembrane protein domain, therefore, a membrane attachment would have to be regulated through post-translational modifications of LARP. This hypothesis was further supported by the use of the H4K20Me3 antibody, which detects methylated lysine residues in proteins. Immunofluorescent assays in this study have shown, that the antibody signal is fully co-localising with the apical accumulation of LARP-GFP. This co-localisation strongly suggests, that LARP is post-translationally methylated, when expressed in the apical region. Recent studies in *Toxoplasma gondii* by the collaborating group of Dominique Soldati-Favre (University of Geneva) has shown that the signal of this antibody is dependent on the presence of the methyl-transferase TgAKMT (Jacot et al., unpublished data). The conditional depletion of the *tgakmt* gene resulted in the complete loss of the apical signal produced by α-H4K20Me3, indicating that TgAKMT is regulating the methylations detected by α-H4K20Me3. Protein lysine methylation is a common post-translational modification of histone proteins in the cell nucleus, in order to regulate gene expression. The *Plasmodium falciparum* orthologue PfSET7 has been shown to modify histone proteins *in vitro* (Chen et al., 2016), however, in *T. gondii* as well as *P. falciparum* the methyltransferase cannot be detected in the nucleus. Therefore, its substrates are more likely to be non-histone proteins, that can be found in the cytosol. Interestingly, TgAKMT has been identified to specifically localise to the apical end of *Toxoplasma gondii* (Heaslip et al., 2011)and a similar pattern of the homologue

PbSET7 can be expected in *Plasmodium berghei*. Until now, no substrates have been identified for this enzyme. The methylation of apically expressed LARP, that is detected through α-H4K20Me3 and the likely apical position of PbSET7 strongly suggest a regulatory connection between these two proteins and indicate LARP as a potential target for PbSET7-methylation. Additionally, the methyltransferase activity has recently been shown to be essential for the invasion and motility of the parasite (Heaslip et al., 2011). A connection between this enzyme and LARP, could be another indicator for a possible involvement of the ARM repeat protein in the motility machinery of Apicomplexan parasites.

Taken together, these results strongly indicate that (i) LARP is post-translationally methylated when localizing to the apical complex and (ii) this post-translational modification could be caused by an interaction with the *P. berghei* orthologue of TgAKMT, PbSET7. However, further experiments and investigations are needed, in order to validate these conclusions. Fluorescence microscopy using either an anti-PbSET7 antibody or a crossed parasite line expressing LARP-GFP and PbSET7-RFP would clarify if these two proteins are truly co-localizing. Through further proteomics and interaction studies (e.g. with BioID as described above) the connection and possible interaction between LARP and PbSET7 can be identified and further characterized.

5.3 Role of LARP in *Plasmodium berghei*

The results obtained and discussed above all indicate a potential connection between LARP and the invasion and motility machinery of *Plasmodium berghei*. In order to determine the function of LARP within the parasite and confirm a potential connection to these processes, different reverse genetic approaches were used during this study. A complete gene knockout of *larp* has shown that asexual blood stages are not viable after transfection of the knockout construct and hence this protein is likely to be essential for the erythrocytic stages of *Plasmodium berghei*.

However, as no drug-resistant parasites were obtained a functional study of other life cycle stages and identification of an exact role of LARP in schizonts and asexual blood stages, was not possible with this approach.

The technique of promoter swap is an established method that has been used in recent research studies to perform a functional analysis of *Plasmodium* genes, that are essential for schizont survival (Sebastian et al., 2012; Siden-Kiamos et al., 2011). By exchanging the endogenous *larp* promoter with an *ama1* promoter a change in the LARP expression pattern was achieved. While the expression remained almost unaltered in schizonts and activated gametocytes, an overexpression of LARP in the motile ookinete stage and a down-regulation in asexual parasite stages was caused by the promoter swap. The down-regulation of *larp* in asexual blood stages, however, has shown no severe defect on the parasite's viability, as the asexual blood stages develop to fully functional and mature schizonts. Despite the distinct patterns of LARP-GFP expression during schizont development (described above), it can therefore be concluded that LARP-GFP is unlikely to play an important role during cell division and schizogony. Similarly, the upregulation of *larp* in ookinetes did not influence the parasite's viability. The development of ookinetes *in vitro* and *in vivo* showed no irregularity and neither the mosquito gut infection nor the sporozoite development were severely impaired. A slight decrease in the mosquito gut infection was detected in LARP-PTD parasites, however these alterations were not significant and could for example be caused by slight differences in the parasitaemia and gametocytaemia of the mouse blood or the mosquito feed.

Therefore, a comprehensive functional analysis of LARP in *Plasmodium berghei* could not be obtained during this study. A third approach to generate a gene depletion through the tetracycline-repressible transactivator system delivered no results, as the transfection of the cloning construct was unsuccessful. However, in recent years many other approaches have been established to analyse

the function of genes and protein in Apicomplexan parasites (de Koning-Ward et al., 2015), that have not been tested in this thesis. When choosing a new knockdown strategy, the possible essentiality of *larp* in the asexual blood stages has to be considered. Therefore, the new system has to either generate an inducible or stage-specific gene knockdown, to secure the survival of parasites immediately after construct transfection. Therefore, strategies like for example CRISPR-Cas9 are not suitable for this approach, as its genome modification is not controllable after transfection and effects every life cycle stage. Potentially, the promoter swap technique could be repeated when a different, more suitable promoter is used. However, the transcript expression of *larp* in ookinetes is extremely low. Therefore, finding another gene that resembles the transcript expression of *larp* in schizonts but is significantly lower in ookinetes is difficult. Therefore, more suitable methods are discussed below.

An inducible post-transcriptional knockdown that has been tested in *Plasmodium* parasites before, uses a ribozyme-based gene expression system (Prommana et al., 2013). Through double homologous recombination a *glmS* ribozyme gets inserted downstream of the gene of interest (GOI), adjacent to the stop codon. The presence of glucosamine-6-phoshate (GlcN-6P) activates the ribozyme after transcription, which cleaves the mRNA, removes the 3' UTR and causes the degradation of the mRNA. Therefore, the expression level of the GOI can be controlled through GlcN-6P on the mRNA level. A post-translational knockdown, that has been used in *Plasmodium* species before is the auxin-inducible Degron (AID) system (Philip and Waters, 2015), which alters the protein rather than the mRNA level. For this approach the F-box protein TIR1, which is usually a plant-specific protein, has to be expressed in the parasite. This protein is a component of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex and responsible for the auxin-dependent recognition of protein substrates through their Degron domain. After recognition of the substrate the protein is degraded by

the proteasome. When the protein of interest is fused with a Degron domain and expressed in the described system, its degradation can be induced by the presence of auxin in the parasite culture. A conditional gene excision in apicomplexan parasites can be achieved through the DiCre system (Andenmatten et al., 2013). The dimerizable Cre recombinase recognises DNA sequences flanked by so-called *loxP* sites (two identical 34 bp sequences) and catalyses their deletion. For this approach the two subunits of DiCre are split into two inactive fragments. The two fragments are fused to FRB or FKBP, two rapamycin-binding proteins. Adding the ligand rapamycin to the parasite induces the dimerization of DiCre and the deletion of the GOI.

These are only three examples, that could be used in order to accomplish a functional analysis of LARP and determine a possible role of this ARM repeat protein within *Plasmodium berghei*. Generating a conditional knockdown system for LARP is essential for the functional characterisation of LARP and would provide the much needed knowledge to further understand the biochemistry of this protein.

5.4 Summary and Outlook

In this study the large Armadillo repeat protein (LARP) was, for the first time, characterised in a *Plasmodium* parasite. It was shown that LARP is expressed throughout the entire *Plasmodium berghei* life cycle and predominantly present in the cytosol. In addition, the invasive stages merozoite, ookinete and sporozoite display a distinct apical localisation of LARP-GFP, that is co-localising with the glideosomal marker protein MyoA. This ring-like, apical accumulation of LARP is likely to be somehow connected to the apical polar ring, as these two structures share a similar localisation, shape and developmental profile during the formation of invasive parasite stages. Furthermore, this work indicates that LARP is attached to the plasma membrane in the apical region of invasive *Plasmodium berghei*

stages. As LARP does not contain transmembrane protein domains, this attachment could be controlled by a post-translational methylation that is regulated by the apical methyltransferase PbSET7.

Taken together the results of this study suggest a role of LARP in the invasion and motility machinery of *Plasmodium berghei*. However, further investigations need to be undertaken for a full characterisation and understanding of this protein. Firstly, the connection of LARP to the apical polar ring needs to be confirmed. APR marker proteins could indicate the localisation of LARP with respect to the apical polar ring in immunofluorescence assays. Further proteomic studies to identify potential interacting partners are needed to understand the biochemistry and dynamics of LARP. In particular, the methyltransferase PbSET7, the structural proteins actin and tubulin as well as other apical proteins need to be tested as potential interaction partners, to confirm the modification, localisation and function of LARP, that have been indicated in this study. To determine the function of LARP within *Plasmodium berghei*, a conditional knockdown has to be generated, for example through methods like the DiCre system, the Degron system or a ribozyme-based gene expression approach.

In summary, this work has highlighted the importance of proteins containing an Armadillo repeat domain in the *Plasmodium* parasite. Focusing more of the future malaria research on these proteins could deliver valuable information on the biology of *Plasmodium* parasites. Understanding how *Plasmodium* parasites invade, divide and survive within their vertebrate host is essential to improve the treatment and prevention of malaria infections. ARM repeat proteins, such as LARP, are likely to play major roles in these pivotal cellular processes and could act as key regulators in securing the parasite’s survival.

6 References

Adams, J.H., Sim, B.K., Dolan, S.A., Fang, X., Kaslow, D.C., and Miller, L.H. (1992). A family of erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci. U. S. A. *89*, 7085–7089.

Afonina, I.S., Elton, L., Carpentier, I., Beyaert, R., Akira, S., Uematsu, S., Takeuchi, O., do Vale, A., Cabanes, D., Sousa, S., et al. (2016). Toxoplasma Profilin Is Essential for Host Cell Invasion and TLR11-Dependent Induction of an Interleukin-12 Response. Nat. Rev. Immunol. *12*, e1005396.

Alavi, Y., Arai, M., Mendoza, J., Tufet-Bayona, M., Sinha, R., Fowler, K., Billker, O., Franke-Fayard, B., Janse, C.J., Waters, A., et al. (2003). The dynamics of interactions between Plasmodium and the mosquito: a study of the infectivity of Plasmodium berghei and Plasmodium gallinaceum, and their transmission by Anopheles stephensi, Anopheles gambiae and Aedes aegypti. Int. J. Parasitol. *33*, 933–943.

Allen, M.L., Dobrowolski, J.M., Muller, H., Sibley, L.D., and Mansour, T.E. (1997). Cloning and characterization of actin depolymerizing factor from Toxoplasma gondii. Mol. Biochem. Parasitol. *88*, 43–52.

Aly, A.S.I., and Matuschewski, K. (2005). A malarial cysteine protease is necessary for *Plasmodium* sporozoite egress from oocysts. J. Exp. Med. *202*, 225–230.

Aly, A.S.I., Vaughan, A.M., and Kappe, S.H.I. (2009). Malaria Parasite Development in the Mosquito and Infection of the Mammalian Host. Annu. Rev. Microbiol. *63*, 195–221.

Amino, R., Thiberge, S., Blazquez, S., Baldacci, P., Renaud, O., Shorte, S., and Ménard, R. (2007). Imaging malaria sporozoites in the dermis of the mammalian host. Nat. Protoc. *2*, 1705–1712.

Anastasiadis, P.Z., and Reynolds, a B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. J. Cell Sci. *113 (Pt 8*, 1319–1334.

Anastasiadis, P.Z., and Reynolds, A.B. (2001). Regulation of Rho GTPases by p120-catenin. Curr. Opin. Cell Biol. *13*, 604–610.

Andenmatten, N., Egarter, S., Jackson, A.J., Jullien, N., Herman, J.-P., and Meissner, M. (2013). Conditional genome engineering in Toxoplasma gondii uncovers alternative invasion mechanisms. Nat. Methods *10*, 125–127.

Baer, K., Klotz, C., Kappe, S.H.I., Schnieder, T., and Frevert, U. (2007). Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. PLoS Pathog. *3*, 1651–1668.

Bannister, L.H., and Dluzewski, A.R. (1990). The ultrastructure of red cell invasion in malaria infections: a review. Blood Cells *16*, 257.

Bannister, L.H., and Mitchell, G.H. (1995). The role of the cytoskeleton in Plasmodium falciparum merozoite biology: an electron-microscopic view. Ann. Trop. Med. Parasitol. *89*, 105–111.

Bannister, L.H., and Sherman, I.W. (2009). *Plasmodium*. Encycl. Life Sci.

Bannister, L.H., Butcher, G. a, Dennis, E.D., and Mitchell, G.H. (1975). Structure and invasive behaviour of Plasmodium knowlesi merozoites in vitro. Parasitology *71*, 483–491.

Bannister, L.H., Mitchell, G.H., Butcher, G.A., Dennis, E.D., and Cohen, S. (1986). Structure and development of the surface coat of erythrocytic merozoites of Plasmodium knowlesi. Cell Tissue Res. *245*, 281–290.

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., and Mitchell, G.H. (2000). Ultrastructure of rhoptry development in Plasmodium falciparum erythrocytic schizonts. Parasitology *121 (Pt 3*, 273–287.

Baton, L. a., and Ranford-Cartwright, L.C. (2004). Plasmodium falciparum ookinete invasion of the midgut epithelium of Anopheles stephensi is consistent with the Time Bomb model. Parasitology *129*, 663–676.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T.W., Green, J.L., Holder, A.A., and Cowman, A.F. (2006a). A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. J. Biol. Chem. *281*, 5197–5208.

Baum, J., Papenfuss, A.T., Baum, B., Speed, T.P., and Cowman, A.F. (2006b). Regulation of apicomplexan actin-based motility. Nat. Rev. Microbiol. *4*, 621–628.

Baum, J., Gilberger, T.-W., Frischknecht, F., and Meissner, M. (2008). Host-cell invasion by malaria parasites: insights from Plasmodium and Toxoplasma. Trends Parasitol. *24*, 557–563.

Beck, J.R., Rodriguez-Fernandez, I.A., de Leon, J.C., Huynh, M.H., Carruthers, V.B., Morrissette, N.S., and Bradley, P.J. (2010). A novel family of Toxoplasma IMC proteins displays a hierarchical organization and functions in coordinating parasite division. PLoS Pathog. *6*.

Beck, J.R., Fung, C., Straub, K.W., Coppens, I., Vashisht, A.A., Wohlschlegel, J.A., and Bradley, P.J. (2013). A Toxoplasma Palmitoyl Acyl Transferase and the Palmitoylated Armadillo Repeat Protein TgARO Govern Apical Rhoptry Tethering and Reveal a Critical Role for the Rhoptries in Host Cell Invasion but Not Egress. PLoS Pathog. *9*.

Bergman, L.W., Kaiser, K., Fujioka, H., Coppens, I., Daly, T.M., Fox, S., Matuschewski, K., Nussenzweig, V., and Kappe, S.H.I. (2003). Myosin A tail domain interacting protein (MTIP) localizes to the inner membrane complex of Plasmodium sporozoites. J. Cell Sci. *116*, 39–49.

Besteiro, S., Dubremetz, J.F., and Lebrun, M. (2011). The moving junction of apicomplexan parasites: A key structure for invasion. Cell. Microbiol. *13*, 797–805.

Billker, O., Lindo, V., Panico, M., Etienne, A.E., Paxton, T., Dell, A., Rogers, M., Sinden, R.E., and Morris, H.R. (1998). Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. Nature *392*, 289–292.

Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004). Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. Cell *117*, 503–514.

Blandin, S., Shiao, S.H., Moita, L.F., Janse, C.J., Waters, A.P., Kafatos, F.C., and Levashina, E.A. (2004). Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. Cell *116*, 661–670.

Boothroyd, J.C., and Dubremetz, J.-F. (2008). Kiss and spit: the dual roles of Toxoplasma rhoptries. Nat. Rev. Microbiol. *6*, 79–88.

Boucher, L.E., and Bosch, J. (2015). The apicomplexan glideosome and adhesins – Structures and function. J. Struct. Biol. *190*, 93–114.

Boyle, M.J., Wilson, D.W., and Beeson, J.G. (2013). New approaches to studying Plasmodium falciparum merozoite invasion and insights into invasion biology. Int. J. Parasitol. *43*, 1–10.

Boyle, M.J., Langer, C., Chan, J.A., Hodder, A.N., Coppel, R.L., Anders, R.F., and Beeson, J.G. (2014). Sequential processing of merozoite surface proteins during and after erythrocyte invasion by Plasmodium falciparum. Infect. Immun. *82*, 924–936.

Brancucci, N.M.B., Bertschi, N.L., Zhu, L., Niederwieser, I., Chin, W.H., Wampfler, R., Freymond, C., Rottmann, M., Felger, I., Bozdech, Z., et al. (2014). Heterochromatin protein 1 secures survival and transmission of malaria parasites. Cell Host Microbe *16*, 165–176.

Buscaglia, C.A., Coppens, I., Hol, W.G.J., and Nussenzweig, V. (2003). Sites of interaction between aldolase and thrombospondin-related anonymous protein in plasmodium. Mol. Biol. Cell *14*, 4947–4957.

Carruthers, V.B. (1999). Armed and dangerous: Toxoplasma gondii uses an arsenal of secretory proteins to infect host cells. Parasitol. Int. *48*, 1–10.

Carruthers, V.B., and Sibley, L.D. (1997). Sequential protein secretion from three distinct organelles of Toxoplasma gondii accompanies invasion of human fibroblasts. Eur. J. Cell Biol. *73*, 114–123.

Chen, A.L., Kim, E.W., Toh, J.Y., Vashisht, A.A., Rashoff, A.Q., Van, C., Huang, A.S., Moon, A.S., Bell, H.N., Bentolila, L.A., et al. (2015). Novel components of the toxoplasma inner membrane complex revealed by BioID. MBio *6*.

Chen, P.B., Ding, S., Zanghì, G., Soulard, V., DiMaggio, P.A., Fuchter, M.J., Mecheri, S., Mazier, D., Scherf, A., and Malmquist, N.A. (2016). Plasmodium falciparum PfSET7: enzymatic characterization and cellular localization of a novel protein methyltransferase in sporozoite, liver and erythrocytic stage parasites. Sci. Rep. *6*, 21802.

Child, M.A., Epp, C., Bujard, H., and Blackman, M.J. (2010). Regulated maturation of malaria merozoite surface protein-1 is essential for parasite growth. Mol. Microbiol. *78*, 187–202.

Choi, H.J., and Weis, W.I. (2005). Structure of the armadillo repeat domain of plakophilin 1. J. Mol. Biol. *346*, 367–376.

Christensen, B.M., Li, J., Chen, C.C., and Nappi, A.J. (2005). Melanization immune responses in mosquito vectors. Trends Parasitol. *21*, 192–199.

Coates, J.C. (2003). Armadillo repeat proteins: Beyond the animal kingdom. Trends Cell Biol. *13*, 463–471.

Coates, J.C. (2008). Armadillo repeat proteins: Versatile regulators of plant development and signalling. Plant Cell Monogr. *10*, 299–314.

Coleman, B.I., Skillman, K.M., Jiang, R.H.Y., Childs, L.M., Altenhofen, L.M., Ganter, M., Leung, Y., Goldowitz, I., Kafsack, B.F.C., Marti, M., et al. (2014). A Plasmodium falciparum Histone Deacetylase regulates antigenic variation and gametocyte conversion. Cell Host Microbe *16*, 177–186.

Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin α. Cell *94*, 193–204.

Coppi, A., Tewari, R., Bishop, J.R., Bennett, B.L., Lawrence, R., Esko, J.D., Billker, O., and Sinnis, P. (2007). Heparan Sulfate Proteoglycans Provide a Signal to Plasmodium Sporozoites to Stop Migrating and Productively Invade Host Cells. Cell Host Microbe *2*, 316–327.

Corish, P., and Tyler-Smith, C. (1999). Attenuation of green fluorescent protein half-life in mammalian cells. Protein Eng. *12*, 1035–1040.

Counihan, N.A., Kalanon, M., Coppel, R.L., and de Koning-Ward, T.F. (2013). Plasmodium rhoptry proteins: why order is important. Trends Parasitol. *29*, 228–236.

Cowman, A.F., and Crabb, B.S. (2002). The Plasmodium falciparum genome--a blueprint for erythrocyte invasion. Science *298*, 126–128.

Cowman, A.F., and Crabb, B.S. (2006). Invasion of red blood cells by malaria parasites. Cell *124*, 755–766.

Cowman, A.F., Berry, D., and Baum, J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. J. Cell Biol. *198*, 961–971.

Daher, W., and Soldati-Favre, D. (2009). Mechanisms controlling glideosome function in apicomplexans. Curr. Opin. Microbiol. *12*, 408–414.

Daniel, J.M., and Reynolds, A.B. (1999). The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol. Cell. Biol. *19*, 3614–3623.

Daniel, J.M., Spring, C.M., Crawford, H.C., Reynolds, A.B., and Baig, A. (2002). The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. Nucleic Acids Res. *30*, 2911–2919.

Delbac, F., S??nger, A., Neuhaus, E.M., Stratmann, R., Ajioka, J.W., Toursel, C., Herm-G??tz, A., Tomavo, S., Soldati, T., and Soldati, D. (2001). Toxoplasma gondii myosins B/C: one gene, two tails, two localizations, and a role in parasite division. J. Cell Biol. *155*, 613–623.

Dobrowolski, J.M., Niesman, I.R., and Sibley, L.D. (1997). Actin in the parasite toxoplasma gondii is encoded by a single copy gene, act1 and exists primarily in a globular form. Cell Motil. Cytoskeleton *37*, 253–262.

Eksi, S., Haile, Y., Furuya, T., Ma, L., Su, X., and Williamson, K.C. (2005). Identification of a subtelomeric gene family expressed during the asexual-sexual stage transition in Plasmodium falciparum. Mol. Biochem. Parasitol. *143*, 90–99.

Fontes, M.R., Teh, T., and Kobe, B. (2000). Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin-alpha. J. Mol. Biol. *297*, 1183–1194.

Foth, B.J., Goedecke, M.C., and Soldati, D. (2006). New insights into myosin evolution and classification. Proc. Natl. Acad. Sci. U. S. A. *103*, 3681–3686.

Francia, M.E., and Striepen, B. (2014). Cell division in apicomplexan parasites. Nat. Rev. Microbiol. *12*, 125–136.

Frevert, U., Sinnis, P., Cerami, C., Shreffler, W., Takacs, B., and Nussenzweig, V. (1993).

Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. J. Exp. Med. *177*, 1287–1298.

Frischknecht, F., Baldacci, P., Martin, B., Zimmer, C., Thiberge, S., Olivo-Marin, J.C., Shorte, S.L., and Ménard, R. (2004). Imaging movement of malaria parasites during transmission by Anopheles mosquitoes. Cell. Microbiol. *6*, 687–694.

Froissard, M., Keller, A.M., and Cohen, J. (2001). ND9P, a novel protein with Armadillo-like repeats involved in exocytosis: Physiological studies using allelic mutants in paramecium. Genetics *157*, 611–620.

Gaskins, E., Gilk, S., DeVore, N., Mann, T., Ward, G., and Beckers, C. (2004). Identification of the membrane receptor of a class XIV myosin in Toxoplasma gondii. J. Cell Biol. *165*, 383–393.

Gerald, N., Mahajan, B., and Kumar, S. (2011). Mitosis in the human malaria parasite plasmodium falciparum. Eukaryot. Cell *10*, 474–482.

Gerold, P., Schofield, L., Blackman, M.J., Holder, A.A., and Schwarz, R.T. (1996). Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of Plasmodium falciparum. Mol. Biochem. Parasitol. *75*, 131–143.

Gilk, S.D., Gaskins, E., Ward, G.E., and Beckers, C.J.M. (2009). GAP45 phosphorylation controls assembly of the Toxoplasma myosin XIV complex. Eukaryot. Cell *8*, 190–196.

Glushakova, S., Yin, D., Li, T., and Zimmerberg, J. (2005). Membrane transformation during malaria parasite release from human red blood cells. Curr. Biol. *15*, 1645–1650.

Gordon, J.L., and Sibley, L.D. (2005). Comparative genome analysis reveals a conserved family of actin-like proteins in apicomplexan parasites. BMC Genomics *6*, 179.

Graham, T.A., Weaver, C., Mao, F., Kimelman, D., and Xu, W. (2000). Crystal Structure of a β-Catenin/Tcf Complex. Cell *103*, 885–896.

Green, J.L., Martin, S.R., Fielden, J., Ksagoni, A., Grainger, M., Yim Lim, B.Y.S., Molloy, J.E., and Holder, A.A. (2006). The MTIP–Myosin A Complex in Blood Stage Malaria Parasites. J. Mol. Biol. *355*, 933–941.

Green, J.L., Rees-Channer, R.R., Howell, S.A., Martin, S.R., Knuepfer, E., Taylor, H.M., Grainger, M., and Holder, A.A. (2008). The Motor Complex of Plasmodium falciparum: PHOSPHORYLATION BY A CALCIUM-DEPENDENT PROTEIN KINASE. J. Biol. Chem. *283*, 30980–30989.

Guttery, D.S., Holder, A.A., and Tewari, R. (2012). Sexual development in Plasmodium: lessons from functional analyses. PLoS Pathog. *8*, e1002404.

Håkansson, S., Morisaki, H., Heuser, J., and Sibley, L.D. (1999). Time-lapse video microscopy of gliding motility in Toxoplasma gondii reveals a novel, biphasic mechanism of cell locomotion. Mol. Biol. Cell *10*, 3539–3547.

Han, Y.S., and Barillas-Mury, C. (2002). Implications of time bomb model of ookinete invasion of midgut cells. In Insect Biochemistry and Molecular Biology, pp. 1311–1316.

Han, Y.S., Thompson, J., Kafatos, F.C., and Barillas-Mury, C. (2000). MC8 - Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: The time bomb theory of ookinete invasion. Mem. Inst. Oswaldo Cruz *95*, 28–29.

Haynes, J.D., Dalton, J.P., Klotz, F.W., McGinniss, M.H., Hadley, T.J., Hudson, D.E., and Miller, L.H. (1988). Receptor-like specificity of a Plasmodium knowlesi malarial protein that

binds to Duffy antigen ligands on erythrocytes. J. Exp. Med. *167*, 1873–1881.

Heaslip, A.T., Nishi, M., Stein, B., and Hu, K. (2011). The motility of a human parasite, Toxoplasma gondii, is regulated by a novel lysine methyltransferase. PLoS Pathog. *7*, e1002201.

Heintzelman, M.B., and Schwartzman, J.D. (1997). A novel class of unconventional myosins from Toxoplasma gondii. J. Mol. Biol. *271*, 139–146.

Heiss, K., Nie, H., Kumar, S., Daly, T.M., Bergman, L.W., and Matuschewski, K. (2008). Functional characterization of a redundant Plasmodium TRAP family invasin, TRAP-like protein, by aldolase binding and a genetic complementation test. Eukaryot. Cell *7*, 1062–1070.

van Hengel, J., Vanhoenacker, P., Staes, K., and van Roy, F. (1999). Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. Proc. Natl. Acad. Sci. U. S. A. *96*, 7980–7985.

Herm-G??tz, A., Weiss, S., Stratmann, R., Fujita-Becker, S., Ruff, C., Meyh??fer, E., Soldati, T., Manstein, D.J., Geeves, M.A., and Soldati, D. (2002). Toxoplasma gondii myosin A and its light chain: A fast, single-headed, plus-end-directed motor. EMBO J. *21*, 2149–2158.

Herold, a, Truant, R., Wiegand, H., and Cullen, B.R. (1998). Determination of the functional domain organization of the importin alpha nuclear import factor. J. Cell Biol. *143*, 309–318.

Hodder, A.N., Drew, D.R., Epa, V.C., Delorenzi, M., Bourgon, R., Miller, S.K., Moritz, R.L., Frecklington, D.F., Simpson, R.J., Speed, T.P., et al. (2003). Enzymic, Phylogenetic, and Structural Characterization of the Unusual Papain-like Protease Domain of Plasmodium falciparum SERA5. J. Biol. Chem. *278*, 48169–48177.

Holder, A.A. (1988). The precursor to major merozoite surface antigens: structure and role in immunity. Prog. Allergy *41*, 72–97.

Huber, A.H., Nelson, W.J., and Weis, W.I. (1997). Three-dimensional structure of the armadillo repeat region of β- catenin. Cell *90*, 871–882.

Huber, M., Cabib, E., and Miller, L.H. (1991). Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. Proc. Natl. Acad. Sci. U. S. A. *88*, 2807–2810.

Huynh, M.H., Rabenau, K.E., Harper, J.M., Beatty, W.L., Sibley, L.D., and Carruthers, V.B. (2003). Rapid invasion of host cells by Toxoplasma requires secretion of the MIC2-M2AP adhesive protein complex. EMBO J. *22*, 2082–2090.

Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2004). Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. PLoS Biol. *2*.

Ishino, T., Chinzei, Y., and Yuda, M. (2005). 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. *7*, 199–208.

Janse, C.J., Mons, B., Rouwenhorst, R.J., Van der Klooster, P.F., Overdulve, J.P., and Van der Kaay, H.J. (1985). In vitro formation of ookinetes and functional maturity of Plasmodium berghei gametocytes. Parasitology *91 (Pt 1)*, 19–29.

Janse, C.J., van der Klooster, P.F.J., van der Kaay, H.J., van der Ploeg, M., and Prosper Overdulve, J. (1986). DNA synthesis in Plasmodium berghei during asexual and sexual development. Mol. Biochem. Parasitol. *20*, 173–182.

Jewett, T.J., and Sibley, L.D. (2003). Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. Mol. Cell *11*, 885–894.

Jiang, J., and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature *391*, 493–496.

Johnson, T.M., Rajfur, Z., Jaconson, K., and Beckers, C.J. (2007). Immobilization of the Type XIV Myosin Complex in Toxoplasma gondii□. Mol. Biol. Cell *18*, 3039–3046.

Ju, D., and Xie, Y. (2006). A synthetic defect in protein degradation caused by loss of Ufd4 and Rad23. Biochem. Biophys. Res. Commun. *341*, 648–652.

Kadota, K., Ishino, T., Matsuyama, T., Chinzei, Y., and Yuda, M. (2004). Essential role of membrane-attack protein in malarial transmission to mosquito host. Proc. Natl. Acad. Sci. U. S. A. *101*, 16310–16315.

Kaiser, K., Camargo, N., Coppens, I., Morrisey, J.M., Vaidya, A.B., and Kappe, S.H.I. (2004). A member of a conserved Plasmodium protein family with membrane-attack complex/perforin (MACPF)-like domains localizes to the micronemes of sporozoites. Mol. Biochem. Parasitol. *133*, 15–26.

Kappe, S., Bruderer, T., Gantt, S., Fujioka, H., Nussenzweig, V., and M??nard, R. (1999). Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. J. Cell Biol. *147*, 937–943.

Kariu, T., Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2006). CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. Mol. Microbiol. *59*, 1369–1379.

Katris, N.J., van Dooren, G.G., McMillan, P.J., Hanssen, E., Tilley, L., and Waller, R.F. (2014). The apical complex provides a regulated gateway for secretion of invasion factors in Toxoplasma. PLoS Pathog. *10*, e1004074.

Keeley, A., and Soldati, D. (2004). The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. Trends Cell Biol. *14*, 528–532.

Kidd, A.R., Miskowski, J.A., Siegfried, K.R., Sawa, H., and Kimble, J. (2005). A β-Catenin Identified by Functional Rather Than Sequence Criteria and Its Role in Wnt/MAPK Signaling. Cell *121*, 761–772.

Kobayashi, N., Yang, J., Ueda, A., Suzuki, T., Tomaru, K., Takeno, M., Okuda, K., and Ishigatsubo, Y. (2007). RanBPM, Muskelin, p48EMLP, p44CTLH, and the armadillo-repeat proteins ARMC8α and ARMC8β are components of the CTLH complex. Gene *396*, 236–247.

Kobe, B. (1999). Autoinhibition by an internal nuclear localization signal revealed bythe crystal structure of mammalian importin |[alpha]|. Nat. Struct. Biol. *6*, 388–397.

de Koning-Ward, T.F., Gilson, P.R., and Crabb, B.S. (2015). Advances in molecular genetic systems in malaria. Nat. Rev. Microbiol. *13*, 373–387.

Korswagen, H.C., Herman, M. a, and Clevers, H.C. (2000). Distinct beta-catenins mediate adhesion and signalling functions in C. elegans. Nature *406*, 527–532.

Koussis, K., Withers-Martinez, C., Yeoh, S., Child, M., Hackett, F., Knuepfer, E., Juliano, L., Woehlbier, U., Bujard, H., and Blackman, M.J. (2009). A multifunctional serine protease primes the malaria parasite for red blood cell invasion. EMBO J. *28*, 725–735.

Lacroix, C., Ménard, R., Heiss, K., al., et, Moreira, C.K., al., et, Sultan, A.A., al., et, Kappe, S., al., et, et al. (2008). TRAP-like protein of Plasmodium sporozoites: linking

gliding motility to host-cell traversal. Trends Parasitol. *24*, 431–434.

Langer, R.C., and Vinetz, J.M. (2001). Plasmodium ookinete-secreted chitinase and parasite penetration of the mosquito peritrophic matrix. Trends Parasitol. *17*, 269–272.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/??-Catenin Signaling: Components, Mechanisms, and Diseases. Dev. Cell *17*, 9–26.

Malik, H.S., Eickbush, T.H., and Goldfarb, D.S. (1997). Evolutionary specialization of the nuclear targeting apparatus. Proc. Natl. Acad. Sci. USA *94*, 13738–13742.

Mason, D.A., Fleming, R.J., and Goldfarb, D.S. (2002). Drosophila melanogaster importin alpha1 and alpha3 can replace importin alpha2 during spermatogenesis but not oogenesis. Genetics *161*, 157–170.

Matz, J.M., Goosmann, C., Brinkmann, V., Grützke, J., Ingmundson, A., Matuschewski, K., and Kooij, T.W. a (2015). The Plasmodium berghei translocon of exported proteins reveals spatiotemporal dynamics of tubular extensions. Sci. Rep. *5*, 12532.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R. V., Rizk, S.S., Njimoh, D.L., Ryan, Y., Chotivanich, K., et al. (2015). A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. Nature *520*, 683–687.

Mehta, S., and Sibley, L.D. (2010). Toxoplasma gondii actin depolymerizing factor acts primarily to sequester G-actin. J. Biol. Chem. *285*, 6835–6847.

Meis, J.F.G.M., and Verhave, J.P. (1988). Exoerythrocytic Development of Malarial Parasites. Adv. Parasitol. *27*, 1–61.

Meis, J.F., Verhave, J.P., Jap, P.H., Sinden, R.E., and Meuwissen, J.H. (1983). Ultrastructural observations on the infection of rat liver by Plasmodium berghei sporozoites in vivo. J. Protozool. *30*, 361–366.

Meissner, M., Schlüter, D., and Soldati, D. (2002). Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. Science *298*, 837–840.

Miller, S.K., Good, R.T., Drew, D.R., Delorenzi, M., Sanders, P.R., Hodder, A.N., Speed, T.P., Cowman, A.F., De Koning-Ward, T.F., and Crabb, B.S. (2002). A subset of Plasmodium falciparum SERA genes are expressed and appear to play an important role in the erythrocytic cycle. J. Biol. Chem. *277*, 47524–47532.

De Monerri, N.C.S., Flynn, H.R., Campos, M.G., Hackett, F., Koussis, K., Withers-Martinez, C., Skehel, J.M., and Blackman, M.J. (2011). Global identification of multiple substrates for Plasmodium falciparum SUB1, an essential malarial processing protease. Infect. Immun. *79*, 1086–1097.

Morrissette, N. (2015). Targeting Toxoplasma Tubules: Tubulin, Microtubules, and Associated Proteins in a Human Pathogen. Eukaryot. Cell *14*, 2–12.

Morrissette, N.S., and Sibley, L.D. (2002). Cytoskeleton of apicomplexan parasites. Microbiol. Mol. Biol. Rev. *66*, 21–38; table of contents.

Mueller, C., Klages, N., Jacot, D., Santos, J.M., Cabrera, A., Gilberger, T.W., Dubremetz, J.F., and Soldati-Favre, D. (2013). The toxoplasma protein ARO mediates the apical positioning of rhoptry organelles, a prerequisite for host cell invasion. Cell Host Microbe *13*, 289–301.

Müller, H.M., Reckmann, I., Hollingdale, M.R., Bujard, H., Robson, K.J., and Crisanti, A. (1993). Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum

binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. EMBO J. *12*, 2881–2889.

Nacer, A., Walker, K., Hurd, H., Guyatt, H., Snow, R., Takken, W., Simonetti, A., Carter, V., Nacer, A., Underhill, B., et al. (2008). Localisation of laminin within Plasmodium berghei oocysts and the midgut epithelial cells of Anopheles stephensi. Parasit. Vectors *1*, 33.

Natarajan, L., Witwer, N.E., and Eisenmann, D.M. (2001). The divergent Caenorhabditis elegans ??-catenin proteins BAR-1, WRM-1 and HMP-2 make distinct protein interactions but retain functional redundancy in vivo. Genetics *159*, 159–172.

Nichols, B.A., and Chiappino, M.L. (1987). Cytoskeleton of Toxoplasma gondii. J. Protozool. *34*, 217–226.

Oakes, R.D., Kurian, D., Bromley, E., Ward, C., Lal, K., Blake, D.P., Reid, A.J., Pain, A., Sinden, R.E., Wastling, J.M., et al. (2013). The rhoptry proteome of Eimeria tenella sporozoites. Int. J. Parasitol. *43*, 181–188.

Osta, M.A., Christophides, G.K., Vlachou, D., and Kafatos, F.C. (2004). Innate immunity in the malaria vector Anopheles gambiae: comparative and functional genomics. J. Exp. Biol. *207*, 2551–2563.

Otto, T.D., Böhme, U., Jackson, A.P., Hunt, M., Franke-Fayard, B., Hoeijmakers, W.A.M., Religa, A.A., Robertson, L., Sanders, M., Ogun, S.A., et al. (2014). A comprehensive evaluation of rodent malaria parasite genomes and gene expression. BMC Biol. *12*, –.

Pace, T., Olivieri, A., Sanchez, M., Albanesi, V., Picci, L., Kiamos, I.S., Janse, C.J., Waters, A.P., Pizzi, E., and Ponzi, M. (2006). Set regulation in asexual and sexual Plasmodium parasites reveals a novel mechanism of stage-specific expression. Mol. Microbiol. *60*, 870–882.

Pan, X., Roberts, P., Chen, Y., Kvam, E., Shulga, N., Huang, K., Lemmon, S., and Goldfarb, D.S. (2000). Nucleus-vacuole junctions in Saccharomyces cerevisiae are formed through the direct interaction of Vac8p with Nvj1p. Mol. Biol. Cell *11*, 2445–2457.

Patra, K.P., and Vinetz, J.M. (2012). New ultrastructural analysis of the invasive apparatus of the plasmodium ookinete. Am. J. Trop. Med. Hyg. *87*, 412–417.

Peifer, M., Berg, S., and Reynolds, A.B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. Cell *76*, 789–791.

Peterson, M.G., Marshall, V.M., Smythe, J.A., Crewther, P.E., Lew, A., Silva, A., Anders, R.F., and Kemp, D.J. (1989). Integral membrane protein located in the apical complex of Plasmodium falciparum. Mol. Cell. Biol. *9*, 3151–3154.

Philip, N., and Waters, A.P. (2015). Conditional degradation of plasmodium calcineurin reveals functions in parasite colonization of both host and vector. Cell Host Microbe *18*, 122–131.

Pino, P., Sebastian, S., Kim, E.A., Bush, E., Brochet, M., Volkmann, K., Kozlowski, E., Llinás, M., Billker, O., and Soldati-Favre, D. (2012). A tetracycline-repressible transactivator system to study essential genes in malaria parasites. Cell Host Microbe *12*, 824–834.

Poulin, B., Patzewitz, E.-M., Brady, D., Silvie, O., Wright, M.H., Ferguson, D.J.P., Wall, R.J., Whipple, S., Guttery, D.S., Tate, E.W., et al. (2013). Unique apicomplexan IMC sub-compartment proteins are early markers for apical polarity in the malaria parasite. Biol. Open *2*, 1160–1170.

Pradel, G., and Frevert, U. (2001). Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. Hepatology *33*, 1154–1165.

Pradel, G., Garapaty, S., and Frevert, U. (2002). Proteoglycans mediate malaria sporozoite targeting to the liver. Mol. Microbiol. *45*, 637–651.

Prommana, P., Uthaipibull, C., Wongsombat, C., Kamchonwongpaisan, S., Yuthavong, Y., Knuepfer, E., Holder, A.A., and Shaw, P.J. (2013). Inducible Knockdown of Plasmodium Gene Expression Using the glmS Ribozyme. PLoS One *8*.

Rees-Channer, R.R., Martin, S.R., Green, J.L., Bowyer, P.W., Grainger, M., Molloy, J.E., and Holder, A.A. (2006). Dual acylation of the 45kDa gliding-associated protein (GAP45) in Plasmodium falciparum merozoites.

Robson, K.J.H., Hall, J.R.S., Jennings, M.W., Harris, T.J.R., Marsh, K., Newbold, C.I., Tate, V.E., and Weatherall, D.J. (1988). A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. Nature *335*, 79–82.

Roux, K.J., Kim, D.I., and Burke, B. (2013). BioID: A screen for protein-protein interactions. Curr. Protoc. Protein Sci.

Russell, D.G., and Burns, R.G. (1984). The polar ring of coccidian sporozoites: a unique microtubule-organizing centre. J. Cell Sci. *65*, 193–207.

Sahoo, N., Beatty, W., Heuser, J., Sept, D., and Sibley, L.D. (2006). Unusual kinetic and structural properties control rapid assembly and turnover of actin in the parasite Toxoplasma gondii. Mol. Biol. Cell *17*, 895–906.

Salmon, B.L., Oksman, a, and Goldberg, D.E. (2001). Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. Proc. Natl. Acad. Sci. U. S. A. *98*, 271–276.

Sanders, P.R., Gilson, P.R., Cantin, G.T., Greenbaum, D.C., Nebl, T., Carucci, D.J., McConville, M.J., Schofield, L., Hodder, A.N., Yates, J.R., et al. (2005). Distinct protein classes including novel merozoite surface antigens in raft-like membranes of Plasmodium falciparum. J. Biol. Chem. *280*, 40169–40176.

Sapiro, R., Tarantino, L.M., Velazquez, F., Kiriakidou, M., Hecht, N.B., Bucan, M., and Strauss, J.F. (2000). Sperm Antigen 6 Is the Murine Homologue of the Chlamydomonas reinhardtii Central Apparatus Protein Encoded by the PF16 Locus. Biol. Reprod. *62*, 511–518.

Schermelleh, L., Heintzmann, R., and Leonhardt, H. (2010). A guide to super-resolution fluorescence microscopy. J. Cell Biol. *190*, 165–175.

Schmitz, S., Grainger, M., Howell, S., Calder, L.J., Gaeb, M., Pinder, J.C., Holder, A.A., and Veigel, C. (2005). Malaria parasite actin filaments are very short. J. Mol. Biol. *349*, 113–125.

Schneider, D., and Shahabuddin, M. (2000). Malaria parasite development in a Drosophila model. Science (80-.). *288*, 2376–2379.

Schüler, H., Mueller, A.K., and Matuschewski, K. (2005a). Unusual properties of Plasmodium falciparum actin: New insights into microfilament dynamics of apicomplexan parasites. FEBS Lett. *579*, 655–660.

Schüler, H., Mueller, A.-K., and Matuschewski, K. (2005b). A Plasmodium actin-

depolymerizing factor that binds exclusively to actin monomers. Mol. Biol. Cell *16*, 4013–4023.

Sebastian, S., Brochet, M., Collins, M.O., Schwach, F., Jones, M.L., Goulding, D., Rayner, J.C., Choudhary, J.S., and Billker, O. (2012). A Plasmodium calcium-dependent protein kinase controls zygote development and transmission by translationally activating repressed mRNAs. Cell Host Microbe *12*, 9–19.

Severo, M.S., and Levashina, E.A. (2014). Mosquito defenses against Plasmodium parasites. Curr. Opin. Insect Sci. *3*, 30–36.

Shen, B., and Sibley, L.D. (2012). The moving junction, a key portal to host cell invasion by apicomplexan parasites. Curr. Opin. Microbiol. *15*, 449–455.

Siden-Kiamos, I., Ganter, M., Kunze, A., Hliscs, M., Steinb??chel, M., Mendoza, J., Sinden, R.E., Louis, C., and Matuschewski, K. (2011). Stage-specific depletion of myosin A supports an essential role in motility of malarial ookinetes. Cell. Microbiol. *13*, 1996–2006.

Sinden, R.E., and Garnham, P.C.C. (1973). A comparative study on the ultrastructure of plasmodium sporozoites within the oocyst and salivary glands, with particular reference to the incidence of the micropore. Trans. R. Soc. Trop. Med. Hyg. *67*, 631–637.

Sinden, R.E., Hartley, R.H., and Winger, L. (1985). The development of Plasmodium ookinetes in vitro: an ultrastructural study including a description of meiotic division. Parasitology *91 (Pt 2)*, 227–244.

Sinden, R.E., Alavi, Y., and Raine, J.D. (2004). Mosquito-malaria interactions: A reappraisal of the concepts of susceptibility and refractoriness. In Insect Biochemistry and Molecular Biology, pp. 625–629.

Singh, S., Plassmeyer, M., Gaur, D., and Miller, L.H. (2007). Mononeme: A new secretory organelle in Plasmodium falciparum merozoites identified by localization of rhomboid-1 protease. Proc. Natl. Acad. Sci. *104*, 20043–20048.

Singh, S., Alam, M.M., Pal-Bhowmick, I., Brzostowski, J.A., and Chitnis, C.E. (2010). Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. PLoS Pathog. *6*.

Sinha, A., Hughes, K.R., Modrzynska, K.K., Otto, T.D., Pfander, C., Dickens, N.J., Religa, A.A., Bushell, E., Graham, A.L., Cameron, R., et al. (2014). A cascade of DNA-binding proteins for sexual commitment and development in Plasmodium. Nature *507*, 253–257.

Smith, E.F., and Lefebvre, P.A. (1996). PF16 encodes a protein with armadillo repeats and localizes to a single microtubule of the central apparatus in Chlamydomonas flagella. J. Cell Biol. *132*, 359–370.

Smith, E.F., and Lefebvre, P.A. (2000). Defining functional domains within PF16: A central apparatus component required for flagellar motility. Cell Motil. Cytoskeleton *46*, 157–165.

Soldati, D., Foth, B.J., and Cowman, A.F. (2004). Molecular and functional aspects of parasite invasion. Trends Parasitol. *20*, 567–574.

Srinivasan, P., Beatty, W.L., Diouf, A., Herrera, R., Ambroggio, X., Moch, J.K., Tyler, J.S., Narum, D.L., Pierce, S.K., Boothroyd, J.C., et al. (2011). Binding of Plasmodium merozoite proteins RON2 and AMA1 triggers commitment to invasion. Proc. Natl. Acad. Sci. U. S. A. *108*, 13275–13280.

Starnes, G.L., Coincon, M., Sygusch, J., and Sibley, L.D. (2009). Aldolase Is Essential for

Energy Production and Bridging Adhesin-Actin Cytoskeletal Interactions during Parasite Invasion of Host Cells. Cell Host Microbe *5*, 353–364.

Stewart, M.J., Schulman, S., and Vanderberg, J.P. (1986). Rhoptry secretion of membranous whorls by Plasmodium falciparum merozoites. Am. J. Trop. Med. Hyg. *35*, 37–44.

Straschil, U., Talman, A.M., Ferguson, D.J.P., Bunting, K.A., Xu, Z., Bailes, E., Sinden, R.E., Holder, A.A., Smith, E.F., Coates, J.C., et al. (2010). The armadillo repeat protein PF16 is essential for flagellar structure and function in Plasmodium male gametes. PLoS One *5*.

Striegl, H., Andrade-Navarro, M.A., and Heinemann, U. (2010). Armadillo motifs involved in vesicular transport. PLoS One *5*.

Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J.-M., Menard, R., and Heussler, V.T. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science *313*, 1287–1290.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K.J.H., Crisanti, A., Nussenzweig, V., Nussenzweig, R.S., and Ménard, R. (1997). TRAP is necessary for gliding motility and infectivity of Plasmodium sporozoites. Cell *90*, 511–522.

Suzuki, T., Ueda, A., Kobayashi, N., Yang, J., Tomaru, K., Yamamoto, M., Takeno, M., and Ishigatsubo, Y. (2008). Proteasome-dependent degradation of alpha-catenin is regulated by interaction with ARMc8alpha. Biochem. J. *411*, 581–591.

Takemae, H., Sugi, T., Kobayashi, K., Gong, H., Ishiwa, A., Recuenco, F.C., Murakoshi, F., Iwanaga, T., Inomata, A., Horimoto, T., et al. (2013). Characterization of the interaction between Toxoplasma gondii rhoptry neck protein 4 and host cellular β-tubulin. Sci. Rep. *3*, 3199.

Tan, K., Duquette, M., Liu, J., Dong, Y., Zhang, R., Joachimiak, A., Lawler, J., and Wang, J. (2002). Crystal structure of the TSP-1 type 1 repeats. J. Cell Biol. *159*, 373–382.

Tarun, A.S., Baer, K., Dumpit, R.F., Gray, S., Lejarcegui, N., Frevert, U., and Kappe, S.H.I. (2006). Quantitative isolation and in vivo imaging of malaria parasite liver stages. Int. J. Parasitol. *36*, 1283–1293.

Tellam, R.L., Wijffels, G., and Willadsen, P. (1999). Peritrophic matrix proteins. Insect Biochem. Mol. Biol. *29*, 87–101.

Tewari, R., Dorin, D., Moon, R., Doerig, C., and Billker, O. (2005). An atypical mitogen-activated protein kinase controls cytokinesis and flagellar motility during male gamete formation in a malaria parasite. Mol. Microbiol. *58*, 1253–1263.

Tewari, R., Bailes, E., Bunting, K.A., and Coates, J.C. (2010). Armadillo-repeat protein functions: Questions for little creatures. Trends Cell Biol. *20*, 470–481.

Thompson, J., Cooke, R.E., Moore, S., Anderson, L.F., Janse, C.J., and Waters, A.P. (2004). PTRAMP; a conserved Plasmodium thrombospondin-related apical merozoite protein. Mol. Biochem. Parasitol. *134*, 225–232.

Tran, J.Q., De Leon, J.C., Li, C., Huynh, M.H., Beatty, W., and Morrissette, N.S. (2010). RNG1 is a late marker of the apical polar ring in Toxoplasma gondii. Cytoskeleton *67*, 586–598.

Treeck, M., Zacherl, S., Herrmann, S., Cabrera, A., Kono, M., Struck, N.S., Engelberg, K., Haase, S., Frischknecht, F., Miura, K., et al. (2009). Functional analysis of the leading

malaria vaccine candidate AMA-1 reveals an essential role for the cytoplasmic domain in the invasion process. PLoS Pathog. *5*.

Van Troys, M., Huyck, L., Leyman, S., Dhaese, S., Vandekerkhove, J., and Ampe, C. (2008). Ins and outs of ADF/cofilin activity and regulation. Eur. J. Cell Biol. *87*, 649–667.

Vanderberg, J., and Rhodin, J. (1967). Differentiation of nuclear and cytoplasmic fine structure during sporogonic development of Plasmodium berghei. J. Cell Biol. *32*, C7–C10.

Vanderberg, J.P., and Frevert, U. (2004). Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes. Int. J. Parasitol. *34*, 991–996.

Vlachou, D., Zimmermann, T., Cantera, R., Janse, C.J., Waters, A.P., and Kafatos, F.C. (2004). Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion. Cell. Microbiol. *6*, 671–685.

Vlachou, D., Schlegelmilch, T., Runn, E., Mendes, A., and Kafatos, F.C. (2006). The developmental migration of Plasmodium in mosquitoes. Curr. Opin. Genet. Dev. *16*, 384–391.

Wang, Y.X., Catlett, N.L., and Weisman, L.S. (1998). Vac8p, a vacuolar protein with armadillo repeats, functions in both vacuole inheritance and protein targeting from the cytoplasm to vacuole. J. Cell Biol. *140*, 1063–1074.

Warburg, a, and Schneider, I. (1993). In vitro culture of the mosquito stages of Plasmodium falciparum. Exp Parasitol *76*, 121–126.

Weis, K., Ryder, U., and Lamond, A.I. (1996). The conserved amino-terminal domain of hSRP1 alpha is essential for nuclear protein import. EMBO J. *15*, 1818–1825.

Wickham, M.E., Culvenor, J.G., and Cowman, A.F. (2003). Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. J. Biol. Chem. *278*, 37658–37663.

Wisse, E., de Zanger, R.B., Charels, K., van der Smissen, P., and McCuskey, R.S. (1985). The liver sieve: Considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of disse. Hepatology *5*, 683–692.

Wu, G., Xu, G., Schulman, B.A., Jeffrey, P.D., Harper, J.W., and Pavletich, N.P. (2003). Structure of a β-TrCP1-Skp1-β-catenin complex: Destruction motif binding and lysine specificity of the SCFβ-TrCP1 ubiquitin ligase. Mol. Cell *11*, 1445–1456.

Xiao, H., El Bissati, K., Verdier-Pinard, P., Burd, B., Zhang, H., Kim, K., Fiser, A., Angeletti, R.H., and Weiss, L.M. (2010). Post-translational modifications to Toxoplasma gondii ??- and ??-tubulins include novel C-terminal methylation. J. Proteome Res. *9*, 359–372.

Yeoh, S., O'Donnell, R.A., Koussis, K., Dluzewski, A.R., Ansell, K.H., Osborne, S.A., Hackett, F., Withers-Martinez, C., Mitchell, G.H., Bannister, L.H., et al. (2007). Subcellular Discharge of a Serine Protease Mediates Release of Invasive Malaria Parasites from Host Erythrocytes. Cell *131*, 1072–1083.

Zhang, J.D., Ruschhaupt, M., and Biczok, R. (2015). ddCt method for qRT – PCR data analysis. Bioconductor 1–8.

Zieler, H., Nawrocki, J.P., and Shahabuddin, M. (1999). Plasmodium gallinaceum ookinetes adhere specifically to the midgut epithelium of Aedes aegypti by interaction with a carbohydrate ligand. J. Exp. Biol. *202*, 485–495.

7 Appendix

Appendix A| pOB277 vector sequence: This vector was used for the generation of a LARP-GFP-tagging construct. The vector contains a *hdhfr* cassette, a *gfp* sequence and an ampicillin resistance. The target sequence was integrated into the vector by using a *KpnI* and *ApaI* restriction site. The vector was originally based on the pDEFhDHPEA vector from Dr. Chris Janse (Leiden University) and modified by Dr. Rita Tewari.

Colour coding as indicated below:

KpnI | *ApaI* | GFP | backbone | hDHFR | backbone

```
1       GGTACCGGGC CCCAGACGTC GGATCCACCG GTCGCCACCA TGGTGAGCAA GGGCGAGGAG
61      CTGTTCACCG GGGTGGTGCC CATCCTGGTC GAGCTGGACG GCGACGTAAA CGGCCACAAG
121     TTCAGCGTGT CCGGCGAGGG CGAGGGCGAT GCCACCTACG GCAAGCTGAC CCTGAAGTTC
181     ATCTGCACCA CCGGCAAGCT GCCCGTGCCC TGGCCCACCC TCGTGACCAC CCTGACCTAC
241     GGCGTGCAGT GCTTCAGCCG CTACCCCGAC CACATGAAGC AGCACGACTT CTTCAAGTCC
301     GCCATGCCCG AAGGCTACGT CCAGGAGCGC ACCATCTTCT TCAAGGACGA CGGCAACTAC
361     AAGACCCGCG CCGAGGTGAA GTTCGAGGGC GACACCCTGG TGAACCGCAT CGAGCTGAAG
421     GGCATCGACT TCAAGGAGGA CGGCAACATC CTGGGGCACA AGCTGGAGTA CAACTACAAC
481     AGCCACAACG TCTATATCAT GGCCGACAAG CAGAAGAACG GCATCAAGGT GAACTTCAAG
541     ATCCGCCACA ACATCGAGGA CGGCAGCGTG CAGCTCGCCG ACCACTACCA GCAGAACACC
601     CCCATCGGCG ACGGCCCCGT GCTGCTGCCC GACAACCACT ACCTGAGCAC CCAGTCCGCC
661     CTGAGCAAAG ACCCCAACGA GAAGCGCGAT CACATGGTCC TGCTGGAGTT CGTGACCGCC
721     GCCGGGATCA CTCTCGGCAT GGACGAGCTG TACAAGTAAG CGGCCGCCGT TTTTCTTACT
781     TATATATTTA TACCAATTGA TTGTATTTAT AACTGTAAAA ATGTGTATGT TGTGTGCATA
841     TTTTTTTTTG TGCATGCACA TGCATGTAAA TAGCTAAAAT TATGAACATT TTATTTTTTG
901     TTCAGAAAAA AAAAACTTTA CACACATAAA ATGGCTAGTA TGAATAGCCA TATTTTATAT
961     AAATTAAATC CTATGAATTT ATGACCATAT TAAAAATTTA GATATTTATG GAACATAATA
1021    TGTTTGAAAC AATAAGACAA AATTATTATT ATTATTATTA TTTTTACTGT TATAATTATG
1081    TTGTCTCTTC AATGATTCAT AAATAGTTGG ACTTGATTTT TAAAATGTTT ATAATGTGAT
1141    TAGCATAGTT AAATAAAAAA AGTTGAAAAA TTAAAAAAAA ACATATAAAC ACAAATGATG
1201    TTTTTTCCTT CAATTTCGAA TTCACTGGCC GTCGTTTTAC AACGTCGTGA CTGGGAAAAC
1261    CCTGGCGTTA CCCAACTTAA TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT
1321    AGCGAAGAGG CCCGCACCGA TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGG
1381    CGCCTGATGC GGTATTTTCT CCTTACGCAT CTGTGCGGTA TTTCACACCG CATATGGTGC
1441    ACTCTCAGTA CAATCTGCTC TGATGCCGCA TAGTTAAGCC AGCCCCGACA CCCGCCAACA
1501    CCCGCTGACG CGCCCTGACG GGCTTGTCTG CTCCCGGCAT CCGCTTACAG ACAAGCTGTG
1561    ACCGTCTCCG GGAGCTGCAT GTGTCAGAGG TTTTCACCGT CATCACCGAA ACGCGCGAGA
1621    CGAAAGGGCC TCGTGATACG CCTATTTTTA TAGGTTAATG TCATGATAAT AATGGTTTCT
1681    TAGACGTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA CCCCTATTTG TTTATTTTTC
1741    TAAATACATT CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAT GCTTCAATAA
1801    TATTGAAAAA GGAAGAGTAT GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT
1861    GCGGCATTTT GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT
1921    GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC
1981    CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA
2041    TGTGGCGCGG TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTCG CCGCATACAC
2101    TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC
2161    ATGACAGTAA GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC
```

```
2221   TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG
2281   GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC
2341   GAGCGTGACA CCACGATGCC TGTAGCAATG GCAACAACGT TGCGCAAACT ATTAACTGGC
2401   GAACTACTTA CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT
2461   GCAGGACCAC TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA
2521   GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC
2581   CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG
2641   ATCGCTGAGA TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA
2701   TATATACTTT AGATTGATTT AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC
2761   CTTTTTGATA ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA
2821   GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG CGTAATCTGC
2881   TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA
2941   CCAACTCTTT TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTCCTT
3001   CTAGTGTAGC CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC
3061   GCTCTGCTAA TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG
3121   TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG
3181   TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG
3241   CATTGAGAAA GCGCCACGCT TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC
3301   AGGGTCGGAA CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT
3361   AGTCCTGTCG GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG
3421   GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT GGCCTTTTGC
3481   TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT
3541   ACCGCCTTTG AGTGAGCTGA TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA
3601   GTGAGCGAGG AAGCGGAAGA GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG
3661   ATTCATTAAT GCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC
3721   GCAATTAATG TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG
3781   GCTCGTATGT TGTGTGGAAT TGTGAGCGGT AAACAATTTC ACACAGGAAA CAGCTTGACC
3841   ATGATTACGC CAAGCTAACA GGCCTCTGCA GCCCAGCTTA ATTCTTTTCG AGCTCTTTAT
3901   GCTTAAGTTT ACAATTTAAT ATTCATACTT TAAGTATTTT TTGTAGTATC CTAGATATTG
3961   TGCTTTAAAT GCTCACCCCT CAAAGCACCA GTAATATTTT CATCCACTGA AATACCATTA
4021   AATTTTCAAA AAAATACTAT GCATATAATG TTATACATAT AAACATAAAA CGCCATGTAA
4081   ATCAAAAAAT ATATAAAAAT ATGTATAAAA ATAAATATGC ACTAAATATA AGCTAATTAT
4141   GCATAAAAAT TAAAGTGCCC TTTATTAACT AGTCGTAATT ATTTATATTT CTATGTTATA
4201   AAAAAATCCT CATATAATAA TATAATTAAT ATATGTAATG TTTTTTTTAT TTTATAATTT
4261   TAATATAAAA TAATATGTAA ATTAATTCAA AAAATAAATA TAATTGTTGT GAAACAAAAA
4321   ACGTAATTTT TTCATTTGCC TTCAAAATTT AAATTTATTT TAATATTTCC TAAAATATAT
4381   ATACTTTGTG TATAAATATA TAAAAATATA TATTTGCTTA TAAATAAATA AAAAATTTTA
4441   TAAAACATAG GGGGATCCAT GGTTGGTTCG CTAAACTGCA TCGTCGCTGT GTCCCAGAAC
4501   ATGGGCATCG GCAAGAACGG GGACCTGCCC TGGCCACCGC TCAGGAACGA ATTTAGATAT
4561   TTCCAGAGAA TGACCACAAC CTCTTCAGTA GAAGGTAAAC AGAATCTGGT GATTATGGGT
4621   AAGAAGACCT GGTTCTCCAT TCCTGAGAAG AATCGACCTT TAAAGGGTAG AATTAATTTA
4681   GTTCTCAGCA GAGAACTCAA GGAACCTCCA CAAGGAGCTC ATTTTCTTTC CAGAAGTCTA
4741   GATGATGCCT TAAAACTTAC TGAACAACCA GAATTAGCAA ATAAAGTAGA CATGGTCTGG
4801   ATAGTTGGTG GCAGTTCTGT TTATAAGGAA GCCATGAATC ACCCAGGCCA TCTTAAACTA
4861   TTTGTGACAA GGATCATGCA AGACTTTGAA AGTGACACGT TTTTTCCAGA AATTGATTTG
4921   GAGAAATATA AACTTCTGCC AGAATACCCA GGTGTTCTCT CTGATGTCCA GGAGGAGAAA
4981   GGCATTAAGT ACAAATTTGA AGTATATGAG AAGAATGATT AAGGATCCCG TTTTTCTTAC
5041   TTATATATTT ATACCAATTG ATTGTATTTA TAACTGTAAA AATGTGTATG TTGTGTGCAT
5101   ATTTTTTTTT GTGCATGCAC ATGCATGTAA ATAGCTAAAA TTATGAACAT TTTATTTTTT
5161   GTTCAGAAAA AAAAAACTTT ACACACATAA AATGGCTAGT ATGAATAGCC ATATTTTATA
5221   TAAATTAAAT CCTATGAATT TATGACCATA TTAAAAATTT AGATATTTAT GGAACATAAT
5281   ATGTTTGAAA CAATAAGACA AAATTATTAT TATTATTATT ATTTTTACTG TTATAATTAT
5341   GTTGTCTCTT CAATGATTCA TAAATAGTTG GACTTGATTT TTAAAATGTT TATAATATGA
5401   TTAGCATAGT TAAATAAAAA AAGTTGAAAA ATTAAAAAAA AACATATAAA CACAAATGAT
5461   GTTTTTTCCT TCAATTTCGA TG
```

Appendix B| pSS368 vector sequence: This vector was used for generating the LARP-PTD construct for a promoter switch. The vector contains two regions homologous to the parasite genome (5' UTR and 3' sequence of the gene of interest) in order to allow double homologous recombination, an *hdhfr* cassette and the *ama1* promoter sequence.

Colour coding as indicated below:

5' GOI-UTR | hDHFR | AMA1 promoter | 3'GOI | backbone

```
1       CCGCGGCATT GAAGAATTGT ATTTTGTTAC AAACAAAAAA ATAAAATAAT GCATATTTGT
61      TTTTGTGCAT TTTTTTTCTT GTATATATAT GTTGTAATAT CAAATATATG ATAGTTTAAT
121     ATGTACCATA TTAAATAGTG ATATCTCTAA AAAAAAAAAA AAAATTATTT ATACATGTAA
181     TATAATAATA TATATTTCAT TTTTGAAGGG GGCGTGAGCA AGCTATATTT TTAAGAACAA
241     AGCTATATCG AATTTTTTTT GAATTTATAA TTTTCTAAAA ATTACATTTA AAATTATGAA
301     TTTTTTTTAA TTAAAAATAT AAAATTTATA TATAATAAAT TTTCTTATAT TTACTATCAT
361     ATAATGTAAG TTTGTGTGAT ATAGATATAT TTATATATTA TATTTTTTTA AATATACAGC
421     TTTTTTTTAC AACAAAGTGT GCGCATAAAA AAATATACAT ATAGTAAAAA AAAAAAAAAA
481     AAAAAAAAAA GCTGGATACT TAAAAAAATA TAAATTTACC TGACCCACGC AAATATGTTA
541     TATTAAATTA TTAACTTTCT ATCGTATTAT TTATTAACTA ATTCATTTGT TACTTTCACA
601     TTCACTCCTT TTTTATTTAC GTTAATTAAA TTAATTATAT TAACATAATA TATTTTTGGC
661     TTTTATTCCT ATATATTTGT TTGTTAAAAA TGTTGTTTTA AGTTATAAAA AAATATAAAA
721     CAAATATATA AATATGTGTA TATAATATAT ATTATATATA TTTGCAACTT TTTATTTGTT
781     CTAGTTCCTT TTTTGTTTTT ATTTATTTTT TGGTTCTTAT ATTATTATAA TTAAAAAAAA
841     AGGTTTTTAA TATTTTTTTG TTTTTTTATT TAATACAGGA ATCTATAATA TACTATATTT
901     TTTAACGTTA TATTTTTAAT TTTGATTTTT CTCGTTTCTC GCTGCAGCCC AGCTTAATTC
961     TTTTCGAGCT CTTTATGCTT AAGTTTACAA TTTAATATTC ATACTTTAAG TATTTTTTGT
1021    AGTATCCTAG ATATTGTGCT TTAAATGCTC ACCCCTCAAA GCACCAGTAA TATTTTCATC
1081    CACTGAAATA CCATTAAATT TTCAAAAAAA TACTATGCAT ATAATGTTAT ACATATAAAC
1141    ATAAAACGCC ATGTAAATCA AAAAATATAT AAAAATATGT ATAAAAATAA ATATGCACTA
1201    AATATAAGCT AATTATGCAT AAAAATTAAA GTGCCCTTTA TTAACTAGTC GTAATTATTT
1261    ATATTTCTAT GTTATAAAAA AATCCTCATA TAATAATATA ATTAATATAT GTAATGTTTT
1321    TTTTATTTTA TAATTTTAAT ATAAAATAAT ATGTAAATTA ATTCAAAAAA TAAATATAAT
1381    TGTTGTGAAA CAAAAAACGT AATTTTTTCA TTTGCCTTCA AAATTTAAAT TTATTTTAAT
1441    ATTTCCTAAA ATATATATAC TTTGTGTATA AATATATAAA AATATATATT TGCTTATAAA
1501    TAAATAAAAA ATTTTATAAA ACATAGGGGG ATCCATGGTT GGTTCGCTAA ACTGCATCGT
1561    CGCTGTGTCC CAGAACATGG GCATCGGCAA GAACGGGGAC CTGCCCTGGC CACCGCTCAG
1621    GAACGAATTT AGATATTTCC AGAGAATGAC CACAACCTCT TCAGTAGAAG GTAAACAGAA
1681    TCTGGTGATT ATGGGTAAGA AGACCTGGTT CTCCATTCCT GAGAAGAATC GACCTTTAAA
1741    GGGTAGAATT AATTTAGTTC TCAGCAGAGA ACTCAAGGAA CCTCCACAAG GAGCTCATTT
1801    TCTTTCCAGA AGTCTAGATG ATGCCTTAAA ACTTACTGAA CAACCAGAAT TAGCAAATAA
1861    AGTAGACATG GTCTGGATAG TTGGTGGCAG TTCTGTTTAT AAGGAAGCCA TGAATCACCC
1921    AGGCCATCTT AAACTATTTG TGACAAGGAT CATGCAAGAC TTTGAAAGTG ACACGTTTTT
1981    TCCAGAAATT GATTTGGAGA AATATAAACT TCTGCCAGAA TACCCAGGTG TTCTCTCTGA
2041    TGTCCAGGAG GAGAAAGGCA TTAAGTACAA ATTTGAAGTA TATGAGAAGA ATGATTAAGG
2101    ATCCCGTTTT TCTTACTTAT ATATTTATAC CAATTGATTG TATTTATAAC TGTAAAAATG
2161    TGTATGTTGT GTGCATATTT TTTTTTGTGC ATGCACATGC ATGTAAATAG CTAAAATTAT
2221    GAACATTTTA TTTTTTGTTC AGAAAAAAAA AACTTTACAC ACATAAAATG GCTAGTATGA
2281    ATAGCCATAT TTTATATAAA TTAAATCCTA TGAATTTATG ACCATATTAA AAATTTAGAT
2341    ATTTATGGAA CATAATATGT TTGAAACAAT AAGACAAAAT TATTATTATT ATTATTATTT
2401    TTACTGTTAT AATTATGTTG TCTCTTCAAT GATTCATAAA TAGTTGGACT TGATTTTTAA
2461    AATGTTTATA ATATGATTAG CATAGTTAAA TAAAAAAAGT TGAAAAATTA AAAAAAAACA
2501    TATAAACACA AATGATGTTT TTTCCTTCAA TTTCGATGGG TACCCCCACA TACATGGATA
2561    TACATATGGT TGTATGAATG TGCATTTTCT ATAAATACGC TATATGCATA ATTATATATT
2621    CCCATGGAAC CAAAATAACA TATTAATGAA TTTTCTTTTA TTGTGAAAAT GTTCTTATTT
2681    GTTTTAAAAA ATCAAAGTAA ATTTAATTCC TTTAATTGTG CTAAATATTG TGTTTGTATA
2741    ACTATAATGA CATTTCCTAT GCTTTTTTAA TATTATATAT AATTTTTTGT GTTTTTTCAA
2801    AAAAAACGTG TATTTGTTTT TATTTGAGTG TACAATTTGC ATAGTGAGTT TAAAGTGTTG
2861    AAAATTATTT AATTTATAAT TTTCATAAAA CGTTTAATCA TTTATCAAAA TTGCGGTTGC
```

```
2921   AATATAGACA GAAAATATTA ACTTAATTTT TCATTGTTTT GTAAAACATT TTCATTATTT
2981   TTTTTAACAA AATTAAAGTA AATGAGATGG TAAATTATTT TAATTATAAC CATTTGTTTA
3041   ATTTTTTTTA TTTTATTACT ATTATTTTTT TTCTTTTGTG CTGCGCAATT AAAAAGAGAA
3101   TTAAGGGTAG CACCAATATA TTTAAGCAAT CTTTTGCGAC ACACAAGAAA AAATATAACA
3161   TCAACATATT TAAAATAATG GAAATTTTTT AATAATTTAT TCTATATATA TTTAATATAT
3221   GTAAAAAAAC TAATATTGTA ATATAATTAA ATAAAACTTG AAATTGATAT AATATGTATA
3281   TATATTGCAT GTTTAGCTTT AAATAATATT TAAGTATTGA TTTTCTTTAC ACACAAAAAC
3341   ATTTGCCTTT TTATGTGAGG ATTATTGTTT CTTTATTTAT ATTGCTATGA ATTTATTCTT
3401   AGTATAAATT TATTTTTTTT CGGCTTTGAA AATAATTTTA TATATATAAA AAGAAAAAAT
3461   ATGGAAAATA AAATTCATAA ATCATATTTG ACACATTTCA ATTTTTTTTT CTTATTTCAC
3521   AGCTAGCCAT ATAATAATAT ATATGTGTCT ATGGGTTCAA ATAGCTTAAT TTCAATGTGC
3581   GTGAGCATTT TTTTTTATAA AAAAACTAAT TTTATGTTTG GTTATGCATG CAACTGTTAT
3641   AAAATAAAAA TTGTAAAATT TTGTTTTTTT TGTACACATA TTTGTACTTT TTTTAATGCA
3701   CATGAAAAAA GAAAGGAAAA AAAAGGGGGA AAAAAAAGGA AAAAAAAAGG GAAAAAAGGG
3781   AAAAAACGTA CATCTACGCA TTGTTATTTA GCAATAAAAT AGAGAACAAA TCTATAAATA
3841   TATAATGTGT ACAACAAATT AATTTAATTT ATATTTTTAC TGTAATTTCA TAATAATTTT
3901   CATTTTTACA TTTTAATGTT TTTTTAATTG GAATATAATT CAAAATGATT TGACACATTT
3961   TACTTATAGA AGAAGGCTAT ATAATTATAT AAGTGTGTAT ATATAAGTAT TGTATGGTAA
4021   TTGTTTTATA AATTAAATTT TTAAAAAACA TTATTCTATT TTATAATTTG TAAATTAAAA
4081   ATATTAATAA AATAAAACGA TATAAAACTC GAGATGGCTG TTACAAATGA GGAATTAAAA
4141   ACAGCCCATA AAATTGTTAG AAGAGTTTCA AATATTGAAG CTTTTGATAA AAGTGGAGTA
4201   GTCTTTAAGG TAATAGAAAT TGCACATAGT ATATTATATA TATATTTATT TATTTATTTT
4261   CGTATTTTTG CCCATTTAAA TTTGTATGTA ATATATTTTT TATTTTATTT TACATTAGGG
4301   ATATCAAATA TGGACAAATA TATCCCCAAC TATAGAGGAA GATCCCAATG TTATGTTTGT
4361   TAAGTGTGTT GTGCAACATG GCTCTAATCA AGATAAATTA AATGTCGTTC AAATTGATCC
4421   ACCTGGAAAC GGAACGGTTA ATAGTCATAA AAAATATATA TATATTTGAA ATAAAACATG
4481   ATAATGAATT GATATCATTG ATATTATAAC ATTACATAAA GGTGTGTATA TAAATAAATA
4541   TATGTTTGTT AATATTTCTT TCTTTTTAGC CATACGAAAT CGACATAAAA AATGCATGGA
4601   ATTGTAACTC ACAAGTAGAC CCTATGTCAT TTGGTGATAT CGGTTTATTA AACCACACAA
4661   ATACACCCTG TGTTCTTGAC TTTTTGAAGC ATAGATATTT AAAGAATCAA ATATACACCA
4721   CTGCCTGTCC TTTAATTGTT GCAATAAACC CATATAAAGA TTTGGGAAAC ACAACTGATG
4781   AATGGATTCG TAAGTATCGT GATGCATCTG ACCACACTAG GTTACCACCC CATATTTTTT
4841   CATGCGCAAG AGAGGCTTTA TCAAATTTAC ATGGCGTTAA CGCGGCCGCA ATTCACTGGC
4901   CGTCGTTTTA CAACGTCGTG ACTGGGAAAA CCCTGGCGTT ACCCAACTTA ATCGCCTTGC
4961   AGCACATCCC CCTTTCGCCA GCTGGCGTAA TAGCGAAGAG GCCCGCACCG ATCGCCCTTC
5021   CCAACAGTTG CGCAGCCTGA ATGGCGAATG GCGCCTGATG CGGTATTTTC TCCTTACGCA
5081   TCTGTGCGGT ATTTCACACC GCATATGGTG CACTCTCAGT ACAATCTGCT CTGATGCCGC
5141   ATAGTTAAGC CAGCCCCGAC ACCCGCCAAC ACCCGCTGAC GCGCCCTGAC GGGCTTGTCT
5201   GCTCCCGGCA TCCGCTTACA GACAAGCTGT GACCGTCTCC GGGAGCTGCA TGTGTCAGAG
5281   GTTTTCACCG TCATCACCGA AACGCGCGAG ACGAAAGGGC CTCGTGATAC GCCTATTTTT
5341   ATAGGTTAAT GTCATGATAA TAATGGTTTC TTAGACGTCA GGTGGCACTT TTCGGGGAAA
5401   TGTGCGCGGA ACCCCTATTT GTTTATTTTT CTAAATACAT TCAAATATGT ATCCGCTCAT
5461   GAGACAATAA CCCTGATAAA TGCTTCAATA ATATTGAAAA AGGAAGAGTA TGAGTATTCA
5521   ACATTTCCGT GTCGCCCTTA TTCCCTTTTT TGCGGCATTT TGCCTTCCTG TTTTTGCTCA
5581   CCCAGAAACG CTGGTGAAAG TAAAAGATGC TGAAGATCAG TTGGGTGCAC GAGTGGGTTA
5641   CATCGAACTG GATCTCAACA GCGGTAAGAT CCTTGAGAGT TTTCGCCCCG AAGAACGTTT
5701   TCCAATGATG AGCACTTTTA AAGTTCTGCT ATGTGGCGCG GTATTATCCC GTATTGACGC
5761   CGGGCAAGAG CAACTCGGTC GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC
5821   ACCAGTCACA GAAAAGCATC TTACGGATGG CATGACAGTA AGAGAATTAT GCAGTGCTGC
5881   CATAACCATG AGTGATAACA CTGCGGCCAA CTTACTTCTG ACAACGATCG GAGGACCGAA
5941   GGAGCTAACC GCTTTTTTGC ACAACATGGG GGATCATGTA ACTCGCCTTG ATCGTTGGGA
6021   ACCGGAGCTG AATGAAGCCA TACCAAACGA CGAGCGTGAC ACCACGATGC CTGTAGCAAT
6081   GGCAACAACG TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA
6141   ATTAATAGAC TGGATGGAGG CGGATAAAGT TGCAGGACCA CTTCTGCGCT CGGCCCTTCC
6201   GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG CGTGGGTCTC GCGGTATCAT
6261   TGCAGCACTG GGGCCAGATG GTAAGCCCTC CCGTATCGTA GTTATCTACA CGACGGGGAG
6321   TCAGGCAACT ATGGATGAAC GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA
6381   GCATTGGTAA CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TAAAACTTCA
6441   TTTTTAATTT AAAAGGATCT AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC
6501   TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA AAGGATCTTC
6561   TTGAGATCCT TTTTTTCTGC GCGTAATCTG CTGCTTGCAA ACAAAAAAAC CACCGCTACC
6621   AGCGGTGGTT TGTTTGCCGG ATCAAGAGCT ACCAACTCTT TTTCCGAAGG TAACTGGCTT
6681   CAGCAGAGCG CAGATACCAA ATACTGTCCT TCTAGTGTAG CCGTAGTTAG GCCACCACTT
6741   CAAGAACTCT GTAGCACCGC CTACATACCT CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC
6801   TGCCAGTGGC GATAAGTCGT GTCTTACCGG GTTGGACTCA AGACGATAGT TACCGGATAA
6861   GGCGCAGCGG TCGGGCTGAA CGGGGGGTTC GTGCACACAG CCCAGCTTGG AGCGAACGAC
6921   CTACACCGAA CTGAGATACC TACAGCGTGA GCATTGAGAA AGCGCCACGC TTCCCGAAGG
6981   GAGAAAGGCG GACAGGTATC CGGTAAGCGG CAGGGTCGGA ACAGGAGAGC GCACGAGGGA
```

```
7041   GCTTCCAGGG GGAAACGCCT GGTATCTTTA TAGTCCTGTC GGGTTTCGCC ACCTCTGACT
7101   TGAGCGTCGA TTTTTGTGAT GCTCGTCAGG GGGGCGGAGC CTATGGAAAA ACGCCAGCAA
7161   CGCGGCCTTT TTACGGTTCC TGGCCTTTTG CTGGCCTTTT GCTCACATGT TCTTTCCTGC
7221   GTTATCCCCT GATTCTGTGG ATAACCGTAT TACCGCCTTT GAGTGAGCTG ATACCGCTCG
7281   CCGCAGCCGA ACGACCGAGC GCAGCGAGTC AGTGAGCGAG GAAGCGGAAG AGCGCCCAAT
7341   ACGCAAACCG CCTCTCCCCG CGCGTTGGCC GATTCATTAA TGCAGCTGGC ACGACAGGTT
7401   TCCCGACTGG AAAGCGGGCA GTGAGCGCAA CGCAATTAAT GTGAGTTAGC TCACTCATTA
7461   GGCACCCCAG GCTTTACACT TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG
7521   TAAACAATTT CACACAGGAA ACAGCTTGAC CATGATTACG CCAAGCTT
```

Appendix C| pOB90 vector sequence: This vector was used for generating the LARP-KO construct, used for *larp* gene depletion. The plasmid contains a *hdhfr* cassette flanked by the 5' and 3' UTR of the GOI.

Colour coding as indicated below:

pBlueScript | HindIII | PbDHFR5'UTR | TgDHFR/TS | PbDHFR3'UTR | EcoRV | pBlueScript

```
1       GTGGCACTTT TCGGGGAAAT GTGCGCGGAA CCCCTATTTG TTTATTTTTC TAAATACATT
61      CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA
121     GGAAGAGTAT GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT
181     GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT
241     TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT
301     TTCGCCCCGA AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA TGTGGCGCGG
361     TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA
421     ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA
481     GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC TTACTTCTGA
541     CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG GATCATGTAA
601     CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA
661     CCACGATGCC TGTAGCAATG GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA
721     CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC
781     TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA GCCGGTGAGC
841     GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG
901     TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA
961     TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT
1021    AGATTGATTT AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA
1081    ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCCGTAG
1141    AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG CGTAATCTGC TGCTTGCAAA
1201    CAAAAAAACC ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTTT
1261    TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC
1321    CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA
1381    TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG TTGGACTCAA
1441    GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG TGCACACAGC
1501    CCAGCTTGGA GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA
1561    GCGCCACGCT TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA
1621    CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG
1681    GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC
1741    TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG
1801    CTCACATGTT CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG
1861    AGTGAGCTGA TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG
1921    AAGCGGAAGA GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCATTAAT
1981    GCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC GCAATTAATG
2041    TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT
2101    TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG
2161    CCAAGCGCGC AATTAACCCT CACTAAAGGG AACAAAAGCT GGGTACCGGG CCCCCCCTCG
2221    AGGTCGACGG TATCGATAAG CTTGCATGCC TGCAGGTCAA CAATAAATAA TAAATAAATA
2281    TTGTGGAAAT AAAATAACAT ATAATTATTT TTAATACATT GATTTCCCTT TTATTTTTTT
2341    AAATTTCATT GATATAAAAA TATATAATAA TAACATATAT GATTTCAAAT TAATCTTTTC
2401    AAAAATGGTG TTTATTTTTG TATGTTTGTG TATGAATTAA TCACATAACA CATCTATTAA
2461    ATTGAGTTGG TAATATAGAC ACAAATAAAT ATATATATTT TTATAGCTTA AAAGTGTGTT
2521    ATGAATATTT TAAGCATATT TTCTTTTTCT TTGGATTGTG TAAAATGAAC TCATATAATG
2581    CGTTTTTTTG TTTTTGTTAT TTTGTCATTT TGTTATTTTG CTATTTTATG GATTAATTTT
2641    TGTTTATAAA ATGGGAAATA ATTTTAACAT ATTTAAATAA ATGGAGAAAA AATATAAAAT
2701    AATTATAAAA AAAAGTTAAT ACACATTTTT TCCTGTTATA GACCTTATAT TTATTTATCC
2761    ATATATATAT ATATATATAT ATATATATAT ATATACATAC CAAGTGAATT AAGAGGAAAG
2821    CTAATTTATT ATTCAGAATA ATATATGAAC TATATATAAT TTTTATTATT TTGGTGTATA
2881    TTAATCTGTC TATATGCATA CATGCAATAA TTTATCGACT TATATATCAA ATAACATAAA
2941    ATAGAAGTGT TTTAAATTAT GGATATATGC TCAATATTCA TTTTTTTTAA TAAGTTAGCT
3001    ATATTTAAAT TATACATTTT ATATATGGTC TCTTTTTTTT TTAAATATTA TTTAAGTGAT
```

```
3061   CATGAAAATA TAAATAATTT TTTTTTATTT AATATCCTTT TGCTTGCATG TGGTAAATGG
3121   AAATTTGGAT GTGTTTTGAA RGTTCGGATA TAGTTGTATG GACATATAAT ATATTTTGTG
3181   AAAAATTGGT TTTATGTTTA TACTTATGCC AATACTTTTT GAGTAAAACA AAGCAAGTGC
3241   TTATAAATAA TTAAAGCCAA TTTTATAATA TATATTTTTT TATTTAATTT GAATTTAGTA
3301   GTATAATTTT TTATGGTAAG TGCTCAAAGA GAGTTGCTTA TAAAGTATGG TTTGTTTCTT
3361   TTTCGCCATT TTGAATTACA CATTAAAAAT ATATAGATAC ATATATTATA ATATGAAATC
3421   ATTAATAATT TAGGGAAATT CTACAAATTT AAAAACGAAT AAAATAATTG TTTTTCATCA
3481   TGCCATAACA CAATATTGAT ATATACATGT ACAAACATTT TTTTTATTTG GAAAATATAA
3541   ATTATATAAA AAAAAATGTA TAGTATACAA AATGAGCATA TTCACACGGG GTGGACGTTC
3601   ATTTTTTCAT TTTTCCCCTG TTTTTTATGA GTATATGATA AAATTTTATG AACATTTACA
3661   CAAAATGAAA ATGGATATAT AGGAAAAATG GAGCGGTATT TCATTTATCT TTGATTGTCA
3721   TTTGGATATT ATATTACCTT GGGTAGGCAA TTAAAAATGT TAAATAACAA TTTAAGGAAA
3781   TTATATTTTA TATATTAAAA TTAACACTGT ATTATATGAT TCGCTTATAA AAGCCACTCT
3841   TTCCCCATGC AAAGCTGTTT AATATCAATT TTAACAAATT ACACACATGT TAATATATTT
3901   ATATATATAA TTTATATATT TATAATTTAT ATATTTATAT TTTTATTATT TATATATTTA
3961   TTATTTATTG TGTGTGTCAA TTCGGGTAGG ATATACCTCT TTTTTATTGT TTAAAGCGAT
4021   TTGTATTCTA AAATATAAAG RATTTGAAAA AGAGAAAGAT AGAATATGAT CCCATCATAT
4081   ATAGCCCTAT AATTTTTATT TAGCAGCGAA TTAATTTTTC TATTAAGTTT ATGTGTAATT
4241   AAAATAACGG AATATATATA ATACAATAAA AAAGTGCATA AATTAAAATT TTTTCAATTA
4301   AATTTTTTTT TTTAAGGGGT TATATAATAT TAAATATATA AAATACGATT ATATATTTTT
4361   GCTACAATTT TTTATATTAA GATATAAATA GTAAATAAAT GGTATTATAT GGCATGTAAT
4421   ATATAAATTT TTTCCAATTT TTATTTTATA TACACTTTTC CTTTTTTTGT CATAAAACTT
4481   AAACAATTTA CACATTCATT TTAAAAATTG ACTATTTGTT TCAACATTTT TTGAGTTTCC
4541   GTTTTATAAT AGTATTTTCA TTTGTATATT GCTTATATAT ATAAATACAC ACCTAAATGT
4601   TACAAAGGAT CAATGCATAA ACCGGTGTGT CTGGTCGTCG CGATGACCCC CAAGAGGGGC
4661   ATCGGCATCA ACAACGGCCT CCCGTGGCCC CACTTGACCA CAGATTTCAA ACACTTTTCT
4721   CGTGTGACAA AAACGACGCC CGAAGAAGCC AGTCGCCTGA ACGGGTGGCT TCCCAGGAAA
4781   TTTGCAAAGA CGGGCGACTC TGGACTTCCC TCTCCATCAG TCGGCAAGAG ATTCAACGCC
4841   GTTGTCATGG GACGGAAAAC CTGGGAAAGC ATGCCTCGAA AGTTTAGACC CCTCGTGGAC
4901   AGATTGAACA TCGTCGTTTC CTCTTCCCTC AAAGAAGAAG ACATTGCGGC GGAGAAGCCT
4961   CAAGCTGAAG GCCAGCAGCG CGTCCGAGTC TGTGCTTCAC TCCCAGCAGC TCTCAGCCTT
5021   CTGGAGGAAG AGTACAAGGA TTCTGTCGAC CAGATTTTTG TCGTGGGAGG AGCGGGACTG
5081   TACGAGGCAG CGCTGTCTCT GGGCGTTGCC TCTCACCTGT ACATCACGCG TGTAGCCCGC
5141   GAGTTTCCGT GCGACGTTTT CTTCCCTGCG TTCCCCGGAG ATGACATTCT TTCAAACAAA
5201   TCAACTGCTG CGCAGGCTGC AGCTCCTGCC GAGTCTGTGT TCGTTCCCTT TTGTCCGGAG
5261   CTCGGAAGAG AGAAGGACAA TGAAGCGACG TATCGACCCA TCTTCATTTC CAAGACCTTC
5321   TCAGACAACG GGGTTCCCTA CGACTTTGTG GTTCTCGAGA AGAGAAGGAA GACTGACGAC
5381   GCAGCCACTG CGGAACCGAG CAACGCAATG AGCTCCTTGA CGTCCACGAG GGAGACAACT
5441   CCCGTGCACG GGTTGCAGGC TCCTTCTTCG GCCGCAGCCA TTGCCCCGGT GTTGGCGTGG
5501   ATGGACGAAG AAGACCGGAA AAAACGCGAG CAAAAGGAAC TGATTCGGGC CGTTCCGCAT
5561   GTTCACTTTA GAGGCCATGA AGAGTTCCAG TACCTTGATC TCATTGCCGA CATTATTAAC
5621   AATGGAAGGA CAATGGATGA CCGAACGGGC GTTGGTGTCA TCTCCAAATT CGGCTGCACT
5681   ATGCGCTACT CGCTGGATCA GGCCTTTCCA CTTCTCACCA CAAAGCGTGT GTTCTGGAAA
5741   GGGGTCCTCG AAGAGTTGCT GTGGTTCATT CGCGGCGACA CGAACGCAAA CCATCTTTCT
5801   GAGAAGGGCG TGAAGATCTG GGACAAGAAT GTGACACGCG AGTTCCTCGA TTCGCGCAAT
5861   CTCCCCCACC GAGAGGTCGG AGACATCGGC CCGGGCTACG GCTTCCAGTG GAGACACTTC
5921   GGCGCGGCAT ACAAAGACAT GCACACAGAC TACACAGGGC AGGGCGTCGA CCAGCTGAAG
5981   AATGTGATCC AGATGCTGAG AACGAATCCA ACAGATCGTC GCATGCTCAT GACTGCCTGG
6041   AATCCTGCAG CGCTGGACGA AATGGCGCTG CCGCCTTGTC ACTTGTTGTG CCAGTTCTAC
6101   GTGAACGACC AGAAGGAGCT GTCGTGCATC ATGTATCAGC GGTCGTGCGA TGTCGGCCTC
6161   GGCGTCCCCT TCAACATCGC TTCCTATTCG CTTTTGACGC TCATGGTTGC ACACGTCTGC
6221   AACCTAAAAC CTAAGGAGTT CATTCACTTC ATGGGGAACA CGCATGTCTA CACGAACCAT
6281   GTCGAGGCTT TAAAAGAGCA GCTGCGGAGA GAACCGAGAC CGTTCCCCAT TGTGAACATC
6341   CTCAACAAGG AACGCATCAA GGAAATCGAC GATTTCACCG CCGAGGATTT TGAGGTCGTG
6401   GGCTACGTCC CGCACGGACG AATCCAGATG GAGATGGCTG TCTAGCGGAA ATACAGAAGC
6461   TAGCTTTGAT CCCGTTTTTC TTACTTATAT ATTTATACCA ATTGATTGTA TTTATAACTG
6521   TAAAAATGTG TATGTTGTGT GCATATTTTT TTTTGTGCAT GCACATGCAT GTAAATAGCT
6581   AAAATTATGA ACATTTTATT TTTTGTTCAG AAAAAAAAAA CTTTACACAC ATAAAATGGC
6641   TAGTATGAAT AGCCATATTT TATATAAATT AAATCCTATG AATTTATGAC CATATTAAAA
6701   ATTTAGATAT TTATGGAACA TAATATGTTT GAAACAATAA GACAAAATTA TTATTATTAT
6761   TATTATTTTT ACTGTTATAA TTATGTTGTC TCTTCAATGA TTCATAAATA GTTGGACTTG
6821   ATTTTTAAAA TGTTTATAAT ATGATTAGCA TAGTTAAATA AAAAAAGTTG AAAAATTAAA
6881   AAAAAACATA TAAACACAAA TGATGTTTTT TCCTTCAATT TCGATATCGA ATTCCTGCAG
6941   CCCGGGGGAT CCACTAGTTC TAGAGCGGCC GCCACCGCGG TGGAGCTCCA ATTCGCCCTA
7001   TAGTGAGTCG TATTACGCGC GCTCACTGGC CGTCGTTTTA CAACGTCGTG ACTGGGAAAA
7061   CCCTGGCGTT ACCCAACTTA ATCGCCTTGC AGCACATCCC CCTTTCGCCA GCTGGCGTAA
7121   TAGCGAAGAG GCCCGCACCG ATCGCCCTTC CCAACAGTTG CGCAGCCTGA ATGGCGAATG
7181   GGACGCGCCC TGTAGCGGCG CATTAAGCGC GGCGGGTGTG GTGGTTACGC GCAGCGTGAC
```

```
7241   CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCTTTCGCT TTCTTCCCTT CCTTTCTCGC
7301   CACGTTCGCC GGCTTTCCCC GTCAAGCTCT AAATCGGGGG CTCCCTTTAG GGTTCCGATT
7361   TAGTGCTTTA CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT CACGTAGTGG
7421   GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT TCTTTAATAG
7481   TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC TCGGTCTATT CTTTTGATTT
7541   ATAAGGGATT TTGCCGATTT CGGCCTATTG GTTAAAAAAT GAGCTGATTT AACAAAAATT
7601   TAACGCGAAT TTTAACAAAA TATTAACGCT TACAATTTAG
```

Appendix D| pPRFp-TRAD4Ty-T7HAPRF-hDHFR vector sequence: This vector was used for generating the LARP-Tet construct, used for the Tetracycline-repressible transactivator system. The vector was obtained from Dr. Paco Pino (University of Geneva). The construct contains an HA-tag for N-terminal gene tagging, the 5' UTR and 5' sequence of the GOI for double homologous recombination and the *trad4* transactivator sequence.

Colour coding as indicated below:

HA-tag | 5'PRF | 5'PRF-UTR | TRAD4 | backbone

```
1      AACTTTCGAT ACCGTCGACC TCGAGTTTAC CACTCCCTAT CAGTGATAGA GAAAAGTGAA
61     AGTCGAGTTT ACCACTCCCT ATCAGTGATA GAGAAAAGTG AAAGTCGAGT TTACCACTCC
121    CTATCAGTGA TAGAGAAAAG TGAAAGTCGA GTTTACCACT CCCTATCAGT GATAGAGAAA
181    AGTGAAAGTC GAGTTTACCA CTCCCTATCA GTGATAGAGA AAAGTGAAAG TCGAGTTTAC
241    CACTCCCTAT CAGTGATAGA GAAAAGTGAA AGTCGAGTTT ACCACTCCCT ATCAGTGATA
301    GAGAAAAGTG AAAGTCGAGC TCGGTACCCA ATTCGCCCTA TAGTGAGTCG CAGCATGCAT
361    GTATTATTCC AATGTGCATG ATAAAAGAAA AAAATAATAT TTATAAAAAA AAAGAAAAAT
421    AAAACAAAAA AAGAAAAAAA AAAAAAAAAA AAAAAAAATA CAAAAATAAA TAATATAATT
481    TATAATTATA TATTCTTGTC ACAATAAAAA TATATATATA TATATATATA TTTATAATAT
541    GTATATTTTA AACTAGAAAA GGAATAACTA ATATTTTATT TATTATCATT CAAGATTTAT
601    ATTTTATAAT AATAAATACC TAATAGAAAT ATATCAGGAT CGCCAGCTGC AGCAAAATGT
661    CAGGTTACCC CTATGACGTG CCCGATTACG CCGGTTACCC CTATGACGTG CCCGATTATG
721    CCGGCATGGA AGAATATTCA TGGGAAAATT TTTTAAATGA CAAACTTTTA GCAACAAATC
781    AAGTTTCTGC TGCTGGATTA GCTTCAGTAT AATAAAACAA TTGAAATAAT ATATATGAAT
841    TGACAATATG ATATATTAGG ACATGCTTTA TTATTATATA CTGTAATGGG TGTATTGCTT
901    AGTTTGAGTG TGCAAGCTCA TATAGCTTTC TCAAATTTCG ATACCCCACC TTTTTATTTA
961    TCATAATACT GTTTTTAAGA TGTATGCTTA TGTGTGTATA CACATATCAA TTTTAATATA
1021   TTTTTTTTTG TATGATATTA TAGGAAGAAG ATGGAGTTGT ATATGAATGC GTAGCAACCC
1081   CCGATGAAAA TAATCCAGAC TTTGATAAAT GGTCTCTTTT TTATAAAGAA GATTATGATA
1141   TAGAAATTGA AGATGAGGTA ATAGATAAAG GATATAATAT TAAATATAAT AAATTAGATT
1201   TTTATCAGAA ATGTTTTACT TTTTATTAAA TGGATTATTA TGAAAGTGTA TTAATATTTA
1261   TGAAGTTTTC AAATTTGATA TATGTGTGTG TGTGAAAATT TTTTATTCTG ATAATAGCTA
1321   GCGTTAAAAT TTTGGACAAA AAAAGCGCAG ATCTGCCGCG GCTTGTTTTA ATTTGTTATT
1381   TTTTGTTGTA TTTATTCAAT TTTTGTAAAT AATAAAAAGT GTGCATGGGT AGAAAAATAT
1441   AGCTCATTAC AGAATAAGCA AATAAACATA ACATGATAAA CAAATATAAC ACGTATAAAT
1501   AAATATGGAT AAAGTAACCT TTTTTTTTAA TAGTGCGTTT GCCTTTGATT TTATGATTTG
1561   CTCTTAAAAA TATCACAATT TTCTCTTTTA AAAATGTCAT AATTTTTCTC TTTGAATTTA
1621   TTATTTACTT AAAAATAATA TTTTTTTGTC GAGGAAAAAA AATAGAAAAA TTTGTTGATA
1681   AGTTTACATA AAACATTTAA AACTCACGAT GTGTGTAAAA GTAGTATATA TTGGTACAAC
1741   TTTTATTAAG ACCGAAATGC ACACTTTTTT TTTCAGCATA TTTTATATAG CACCTAACAA
1801   AAACAGATAA TAAATATAAT GTTTTAAGTT TAAAATTTGT AAATGTAATT TATTCATGCA
1861   TTTGCAAATT TATGCATTTG ATCGGCGTAA GGCTTCGTCC GTTTTTCACT TTTATTTATT
1921   TTATTTTAAT TTTTTATTTC ATTTTATTTT ATTTATTTTT TGCTCTTTTA AAAAGTAATA
1981   AATAAACTAT TAATGCTTCC CTTTCCTCAT ATAATATATT TTAAGCCCTC CGCGGAAGCT
2041   TAAAATGTCG CGCCTGGACA AGAGCAAAGT CATCAACTCT GCTCTGGAAT TACTCAATGA
2101   AGTCGGTATC GAAGGCCTGA CGACAAGGAA ACTCGCTCAA AAGCTGGGAG TTGAGCAGCC
2161   TACCCTGTAC TGGCACGTGA AGAACAAGCG GGCCCTGCTC GATGCCCTGG CAATCGAGAT
2221   GCTGGACAGG CATCATACCC ACTTCTGCCC CCTGGAAGGC GAGTCATGGC AAGACTTTCT
2281   GCGGAACAAC GCCAAGTCAT TCCGCTGTGC TCTCCTCTCA CATCGCGACG GGGCTAAAGT
2341   GCATCTCGGC ACCCGCCCAA CAGAGAAACA GTACGAAACC CTGGAAAATC AGCTCGCGTT
2401   CCTGTGTCAG CAAGGCTTCT CCCTGGAGAA CGCACTGTAC GCTCTGTCCG CCGTGGGCCA
2461   CTTTACACTG GGCTGCGTAT TGGAGGATCA GGAGCATCAA GTAGCAAAAG AGGAAAGAGA
2521   GACACCTACC ACCGATTCTA TGCCCCCACT TCTGAGACAA GCAATTGAGC TGTTCGACCA
2581   TCAGGGAGCC GAACCTGCCT TCCTTTTCGG CCTGGAACTA ATCATATGTG GCCTGGAGAA
2641   ACAGCTGAAG TGCGAAAGCG GTATGCATGA GGTCCACACG AACCAGGACC CGCTCGACGC
2701   GAACAACATG AACCACATCC CGTACTACAA CGACATGAAT AACAATATCA CGAACTACGC
```

```
2761   GAACATGACG GGCATCCAGA ACCTCAACAA TATGAACCCG ATCATGATTG ACCCTAACAA
2821   CAACGCGGAC TCTGTCGAGG AATTCTACGA CGCGATCGAC GGCTGCAACG TCGTCGACAC
2881   GAACTACATG GTCGGCACAA ACAACATGAT TGACCAGATC CACTTCAACG ACGTCACGTC
2941   TAACGTCGGC AACATGAACA ACAACTGATT AATTAATCAC CTAGGGTCGA GGGATATGGC
3001   AGCTTAATGT TCGTTTTTCT TATTTATATA TTTATACCAA TTGATTGTAT TTATAACTGT
3061   AAAAATGTGT ATGTTGTGTG CATATTTTTT TTTGTGCATG CACATGCATG TAAATAGCTA
3121   AAATTATGAA CATTTTATTT TTTGTTCAGA AAAAAAAAAC TTTACACACA TAAAATGGCT
3181   AGTATGAATA GCCATATTTT ATATAAATTA AATCCTATGA ATTTATGACC ATATTAAAAA
3241   TTTAGATATT TATGGAACAT AATATGTTTG AAACAATAAG ACAAAATTAT TATTATTATT
3301   ATTATTTTTA CTGTTATAAT TATGTGTCTC CTTCAATGAT TCATAAATAG TTGGACTTGA
3361   TTTTTAAAAT GTTTATAATA TGATTAGCAT AGTTAAATAA AAAAAGTTGA AAAATTAAAA
3421   AAAAACATAT AAACACAAAT GATGGTTTTT CCTTCAATTT CGATATCATA TAGCAATCGT
3481   ATTAGCAGGT AAATGAGCAA GGTTAATAAA AAAATGGTAA AAAAAAAAAA TTGGGGAAAA
3541   ATAAACAAGG TGTCTTTATC TACACTTTGG CTTTACAATG TATAATTTAT ATGCATACAA
3601   AAAAATTTTG ATAAAATTGT AAAAAGGTGA ATAAAATAAC ATTGTAACAG TAGTAGATAG
3661   TAAAGGGAAG GTGTTGCTCA AATAGTGTCG AAACAAAACT GGCATAAAGA AAAATTGATA
3721   TTGAGCAGAG GATATGCGCA TAATGGTATT TTGTTTGTTT GTTTGTTTTC TCATTTTTTG
3781   AGACAGCTCA ATTCTTTATG TCCACAACAT CATCGGACTT TTCTTCTTCA GGGTAGGCGG
3841   CCGCTTAATC ATTCTTCTGA GTATTCTATA GTGTCACCTA AATAGCTTGG CGTAATCATG
3901   GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC
3961   CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC
4021   GTTGCGCTCA CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT
4081   CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC TCTTCCGCTT CCTCGCTCAC
4141   TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT
4201   AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA
4261   GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCGATA GGCTCCGCCC
4321   CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT
4381   ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT
4441   GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCATAG
4501   CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA
4561   CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA
4621   CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC
4681   GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG
4741   AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG
4801   TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA
4861   GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC
4921   TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG
4981   GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA
5041   TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT
5101   CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG
5161   GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC
5221   TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC
5281   AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC
5341   GCCAGTTAAT AGTTTGCGCA ACGTTGTTGG CATTGCTACA GGCATCGTGG TGTCACGCTC
5401   GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC
5461   CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA
5521   GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT
5581   GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA
5641   GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA
5701   TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG
5761   GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC
5821   AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC
5881   AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCAATA
5941   TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA
6001   GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA
6061   AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG
6121   TCTCGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC CGGAGACGGT
6181   CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC CGTCAGGGCG CGTCAGCGGG
6241   TGTTGGCGGG TGTCGGGGCT GGCTTAACTA TGCGGCATCA GAGCAGATTG TACTGAGAGT
6301   GCACCATATG CGGTGTGAAA TACCGCACAG ATGCGTAAGG AGAAAATACC GCATCAGGCG
6361   CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA TCGGTGCGGG CCTCTTCGCT
6421   ATTACGCCAG CTGGCGAAAG GGGGATGTGC TGCAAGGCGA TTAAGTTGGG TAACGCCAGG
6481   GTTTTCCCAG TCACGACGTT GTAAAACGAC GGCCAGTGAA TTGTAATACG ACTCACTATA
6541   GAATTCTAGA TTTAATAAAT ATGTTCTTAT ATATAATGAG AAATAAATAT TTAACATATG
6601   TTTAAAAAGA AAAATTTAAG ATTTACATGA TTAGGAATAA AAATATTAAA AGCTATATAT
6661   TAATGTTGTA CTTTATGTTC ATATTGTAAA AGATAAATAT AATTTTTATT AAAATTTATA
6721   TTTATTATTA TTATAAGTTC TTAGAAAATA CATTTTGTTT TAAAATAATA CTATAAAAAG
6781   AACAATAATA AAAATAAAAT ACAATTAAAA CATAATTAAA AAAGAAAAAT ACAGACAGTA
```

```
6841   AAAAAAATCG CTATCCCATA AATTACAAAA CATGAATTAA TAAACATATA TTGTTTATTA
6901   ATAAAATTGT AAGTTATTTT TATATATACA TAATAAATTA AAGATACTGA AACAATAATT
6961   TTAAATGTAA ATAAATGAAT AATGATATGT TTTATATGAT TCATTATTCT ATATTTATAA
7021   GGAAGATTAC AAAAAAAAAA TTCATATGTA TTTTTTTTGT AATTTCTGTG TTTATGTTTT
7081   TATTTTTATA ATATTTTTAA TCTATTATTA AATAAGCTTA ATCATTCTTC TCATATACTT
7141   CAAATTTGTA CTTAATGCCT TTCTCCTCCT GGACATCAGA GAGAACACCT GGGTATTCTG
7201   GCAGAAGTTT ATATTTCTCC AAATCAATTT CTGGAAAAAA CGTGTCACTT TCAAAGTCTT
7261   GCATGATCCT TGTCACAAAT AGTTTAAGAT GGCCTGGGTG ATTCATGGCT TCCTTATAAA
7321   CAGAACTGCC ACCAACTATC CAGACCATGT CTACTTTATT TGCTAATTCT GGTTGTTCAG
7381   TAAGTTTTAA GGCATCATCT AGACTTCTGG AAAGAAAATG AGCTCCTTGT GGAGGTTCCT
7441   TGAGTTCTCT GCTGAGAACT AAATTAATTC TACCCTTTAA AGGTCGATTC TTCTCAGGAA
7501   TGGAGAACCA GGTCTTCTTA CCCATAATCA CCAGATTCTG TTTACCTTCT ACTGAAGAGG
7561   TTGTGGTCAT TCTCTGGAAA TATCTAAATT CGTTCCTGAG CGGTGGCCAG GGGTAGTCCC
7621   CGTTCTTGCC GATGCCCATG TTCTGGGACA CAGCGACGAT GCAGTTTAGC GAACCATGCA
7681   TGGATCCTGA TATATTTCTA TTAGGTATTT ATTATTATAA AATATAAATC TTGAATGATA
7741   ATAAATAAAA TATTAGTTAT TCCTTTTCTA GTTTAAAATA TACATATTAT AAATATATAT
7801   ATATATATAT ATATTTTTAT TGTGACAAGA ATATATAATT ATAAATTATA TTATTTATTT
7861   TTGTATTTTT TTTTTTTTTT TTTTTTTTTT CTTTTTTTGT TTTATTTTTC TTTTTTTTTA
7921   TAAATATTAT TTTTTTCTTT TATCATGCAC ATTGGAATAA TACATTAATA TATATATATA
7981   TATTATATTA TACATATATT GAATAATGTT TATAAAAAAT GCATAACTTA TATGAATATA
8041   ATTTTTTTTA AATATGACAA AAAGAAAAAA AAAAAAAACC AAAAAAAATT AAAATTGAAA
8101   TGAAATATAT AAATATATTA TTTATATATA TTATACATTG TTTAATACTA CTACATGTAT
8161   ATATATATAT TATATATATA TATATATATC AATTTTTTCA AAAATAAATT AATATAAAAA
8241   GAGGGGAAAA AAAAAAAAAA AAAAAAAAAA AGATAATTAA GTAAGCATTT AAAAATATAT
8301   AAATTGATAA TATATAAAAT TAATCACATA TAAACTAATA TAATTTATAA AATAAGGAAA
8361   ATAAAATATT ACCATAAAAT AAAAATAAAA ATAAAAAAAA AAAAAAAAAA CACCTTTTTT
8421   TATATATATT AATATATAAT TATCTCTTAG AAAAAATATT GTATAATTAT ATATGTAATG
8481   ATTTATATAA AAAAATAAAA TTATACAAGT ATATATTTTG TTTCTATAAA TTGATATCTT
8541   AATTATTTAT TATTAGAAAT AGATATTTTT ATAATAAACC AATAGATAAA ATTTGTAGAG
8601   AAAAAAAAAT AAAAATAAAA ATAAAAATAA TATAATATAT AATAAAATAA AATAATATTA
8661   TATAAATATA TTTTAATTTT TTTTACAAAA TGGTT
```

Appendix E| Calculation of qRT-PCR results: The qRT-PCR results were calculated by relativizing the C_T-values to reference genes. Results of qRT-PCR generated from mutant parasite strains were analysed using the $\Delta\Delta C_T$ method.

Steps to calculate the target gene expression in a mutant strain relative to a WT strain:

1. Calculation of the ΔC_T value:

$$\Delta C_T = C_T(target) - C_T(reference)$$

2. Calculation of the $\Delta\Delta C_T$ value:

$$\Delta\Delta C_T = \Delta C_T(Mutant) - \Delta C_T(Wildtype)$$

3. Calculation of relative gene expression:

$$Relative\ gene\ expression = 2^{-\Delta\Delta C_T}$$

4. Calculation of target gene expression in a WT strain:

$$WT\ gene\ expression = 2^{-\Delta C_T}$$

Appendix F| Phenotypic analysis of LARP promoter switch: Raw data obtained from the phenotypic analysis of the LARP-PTD. Asexual blood stages and gametocytes were analysed through Giemsa stained blood smears. Ookinetes were investigate after incubation in ookinete culture for 24h. Oocyst and sporozoites were obtained 14 and 21 days post infection.

Parasite counts in Giemsa stained tail blood smears

Sample	mouse	RBC	Rings	Troph	Gam.	Shz	total	Parasite [%]	Gam. [%]
WT	1	700	116	43	46	10	915	23.50	5.03
	2	698	127	42	65	3	935	25.35	6.95
	3	691	113	87	34	1	926	25.38	3.67
	4	678	114	71	54	2	919	26.22	5.88
PTD	5	682	127	78	57	3	947	27.98	6.02
	6	701	110	60	40	3	914	23.30	4.38
	7	669	154	68	28	2	921	27.36	3.04
	8	699	113	84	31	3	930	24.84	3.33

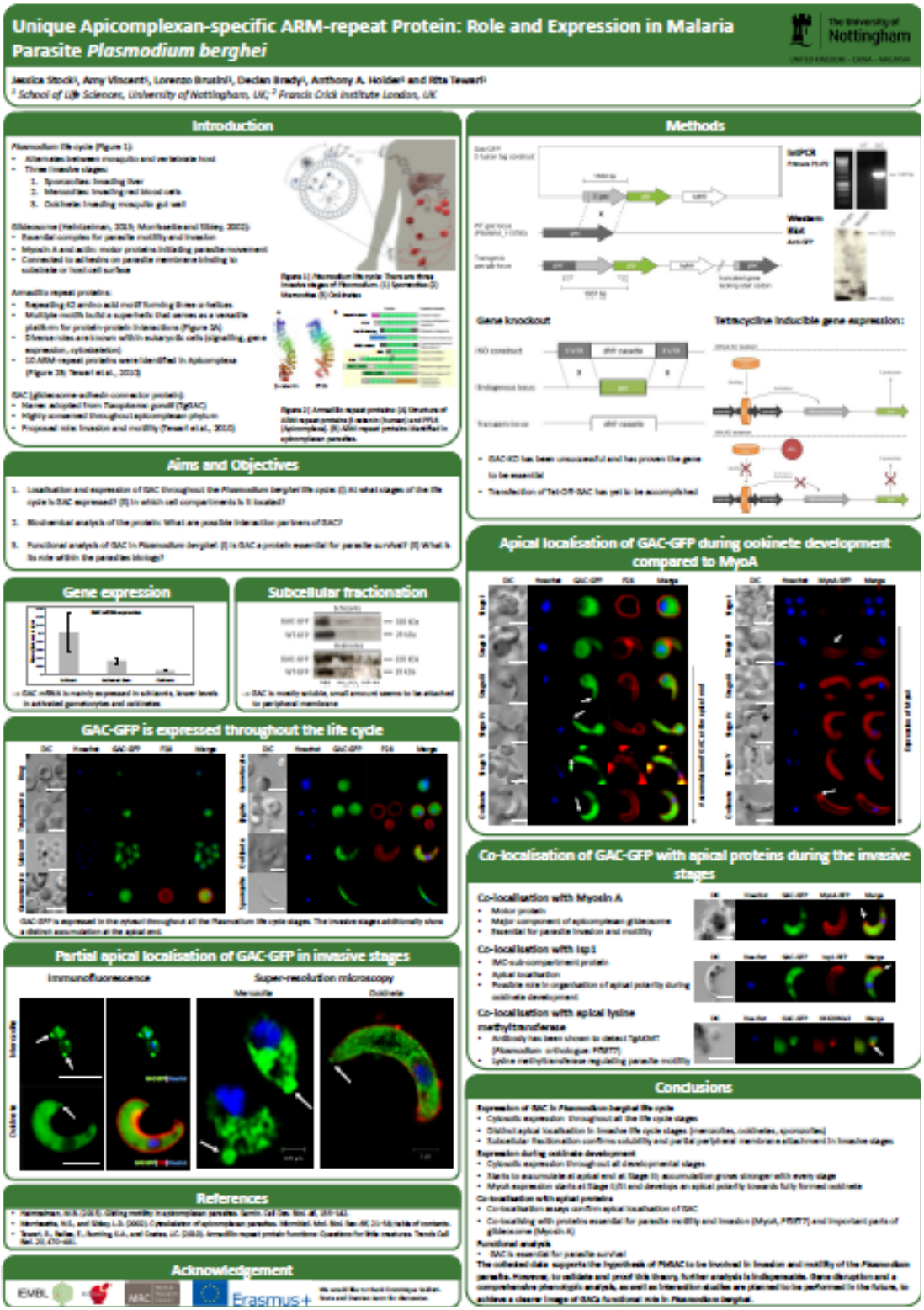
Counts of merozoites per schizont

		WT					PTD			
Mouse		1	2	3	4	5	6	7	8	9	10
Ring		32	29	24	41	31	28	28	30	27	32
Troph		210	244	196	204	209	201	220	212	179	194
Shz		103	104	80	89	94	101	101	94	113	95
Merozoite per shz	1	16	13	9	13	15	14	14	11	14	12
	2	13	15	12	13	13	18	10	12	12	10
	3	12	15	16	15	12	20	14	13	20	13
	4	15	18	15	11	15	19	9	15	18	11
	5	18	16	13	20	15	12	12	11	16	15
	6	17	14	15	12	11	20	12	16	13	12
	7	12	10	14	10	19	12	13	17	15	14
	8	12	11	16	11	13	23	21	12	16	12
	9	20	13	15	14	15	11	15	11	12	16
	10	12	15	14	13	12	16	14	17	13	15
	11	13	14	21	11	12	10	18	13	16	12
	12	11	21	13	16	15	19	13	10	12	12
	13	14	16	17	15	12	14	14	12	20	13
	14	10	16	14	13	12	12	15	13	16	12
	15	12	12	15	12	16	18	14	12	15	11
	16	14	11	13	14	17	17	12	11	16	10
	17	13	14	17	18	17	16	11	13	9	12
	18	9	17	10	11	11	13	11	11	10	13
	19	13	12	12	9	12	11	12	18	22	11
	20	14	13	16	9	11	15	17	13	14	17

Ookinete conversion counts

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
Sample	WT	N6PTD	WT	N6PTD	WT	N6PTD	WT	N6PTD
Exflagallation			8	6	3	2	5	5
			7	7	2	2	7	5
			8	6	4	1	8	10
			7	5	3	1	10	7
			7	7	3	3	6	8
			5	8	3	1	7	10
			7	6	1	2	8	5
					2	1	6	4
					3	2	6	7
Average			7	6	3	2	7	7
Ookinete	118	76	167	150	98	104	147	156
Zygote	117	54	59	76	96	101	120	120
Retorts			4	10	28	19	17	29
Conversion [%]	50.21	58.46	72.61	63.56	44.14	46.43	51.76	51.15

Appendix G| Project poster presented at EMBL conference: BioMalPar XII



Appendix H| Manuscript extract for paper submitted to "Cell Host and Microbe" including results obtained in this study

The connector between glideosomes and adhesins regulates actin dynamics and integrates the lipid signal that regulates invasion in Apicomplexa

Authors

Damien Jacot[1], Nicolo Tosetti[1], Isa Pires[2], Jessica. Stock[3], Arnault. Graindorge[1], Yu-Fu Hung[2], Rita Tewari[3], Inari Kursula[2,4*], Dominique Soldati-Favre[1*]

Affiliations

[1]Department of Microbiology & Molecular Medicine, University of Geneva, 1 Rue Michel-Servet, 1211 Geneva, Switzerland.

[2]Biocenter Oulu and Faculty of Biochemistry and Molecular Medicine, University of Oulu, Aapistie 7, 90220 Oulu, Finland.

[3]School of Life Sciences, Queens Medical Centre, University of Nottingham, NG2 7UH, UK.

[4] Department of Biomedicine, University of Bergen, Jonas Lies vei 91, 5009 Bergen, Norway.

*Correspondence to Dominique.Soldati-Favre@unige.ch or Inari.Kursula@uib.no